CONGENITAL CATARACT IN ALBINO RATS FED DIFFERENT AMOUNTS OF TRYPTOPHAN AND NIACIN[1]

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FIVE FIGURES

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Many reports have appeared in the literature concerning the production of tryptophan deficiency cataracts in the albino rat (Totter and Day, '41, '42; Albanese and Buschke, '42; Schaeffer and Geiger, '47; Schaeffer and Murray, '50). The present study was undertaken to determine whether the limitation of tryptophan in the maternal diet during the gestation period could cause the appearance of congenital cataracts. The findings of Warkany and Schraffenberger ('44, '46) suggested that this might occur, since they demonstrated that the limitation of riboflavin or of vitamin A in the maternal diet can lead to the production of congenital malformations.

The evidence presented will show that a tryptophan-deficient diet such as that used by Albanese and Buschke ('42) can induce congenital cataract, but that this cataract is not

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eliminated by the addition of tryptophan alone at a level which has been considered adequate for the growing rat. However, the simultaneous addition of niacin at this level of tryptophan prevented the development of cataract.

EXPERIMENTAL METHOD

Female rats of the Sprague-Dawley strain were maintained on a diet of laboratory chow until they attained a weight of approximately 150 gm. Estrous cycles were followed by daily vaginal smears and mating was confirmed by the presence of sperm in the smear. The same group of male rats was used for mating with both experimental and control animals.

On the morning mating was confirmed, the experimental animals were placed on one of the 7 diets shown in table 1. The basal diet used in this experiment was selected, in spite of the possibility that it might also be deficient in factors other than tryptophan, because its employment by other investigators (Totter and Day, '42; Albanese and Buschke, '42; Buschke, '43) had provided the bulk of the published data on tryptophan-deficiency cataract. Niacin was added to one of the two diets employing the high tryptophan level (diet A), and to all the diets in which the tryptophan level was reduced, in order to avoid inducing a niacin deficiency at these lowered levels of intake. Both supplements were incorporated into the diet and the diet was fed ad libitum. A group of control animals was continued on the laboratory chow (diet R) throughout the gestation period. Daily weight gains of the animals were recorded.

The morning sperm were found in the vaginal smear was considered to be the first day of pregnancy, and on the 22nd day of the gestation period the young were removed by abdominal section. Six hundred young comprising 84 litters were obtained. Half of each litter was fixed in 10% formalin in physiological saline and prepared for histological examination; the other half was fixed in alcohol and subsequently

cleared for skeletal examination according to the Schultze-Dawson technique.

A random selection of heads of at least 10 fetuses representing a minimum of 7 litters obtained from mothers on each of the two high tryptophan diets (A and I), the two lowest tryptophan diets (E and F), and the control diet (R) were cut along the transverse axis in serial sections, 10 μ in thickness, and stained with hematoxylin and eosin.[4] Due to the

TABLE 1

Composition of experimental diets

BASAL RATION	AMOUNT	SUPPLEMENTS		
		Diet	Tryptophan	Niacin
	gm		*gm %*	*mg %*
Casein, acid-hydrolyzed [1]	14.7	A	0.2	10
Sucrose	15.0	I	0.2	
Cornstarch	42.0	B	0.1	10
Yeast, brewers'	4.3	C	0.075	10
Salt mixture no. 2 [2]	2.0	D	0.050	10
Agar	2.0	E	0.025	10
Crisco	19.0	F	0.010	10
Cod liver oil	5.0			
l-Cystine	0.15			

[1] Supplied by Sterling-Winthrop Research Institute.

[2] Osborne, T. B., and L. B. Mendel. J. Biol. Chem., *54:* 131, 1918.

costliness of preparing serial sections, no representatives of the young obtained from mothers on the intermediate levels of tryptophan (B, C and D) were cut for this study.

The findings reported in this paper are concerned with the condition of the maternal rats and the histological findings in the fetal eyes.

RESULTS AND DISCUSSION

The inadequacy of all of the tryptophan-supplemented acid-hydrolyzed casein diets was readily apparent from the condition of the animals during the gestation period. Although

[4] The technical assistance of Marelene Cox is gratefully acknowledged.

no effort was made to restrict the food intake, it was noted that intakes declined as the content of the essential amino acid, tryptophan, decreased in the ration. This finding has been reported by other investigators (Cox and Mueller, '39; Frazier et al., '47). The decline in food intake was paralleled by a lessened weight gain in the mothers during the pregnancy period (fig. 1). Even on those diets in which the tryptophan supplement was 0.2%, the maternal weight gain approximated only 45% of the weight gain of the animals on the

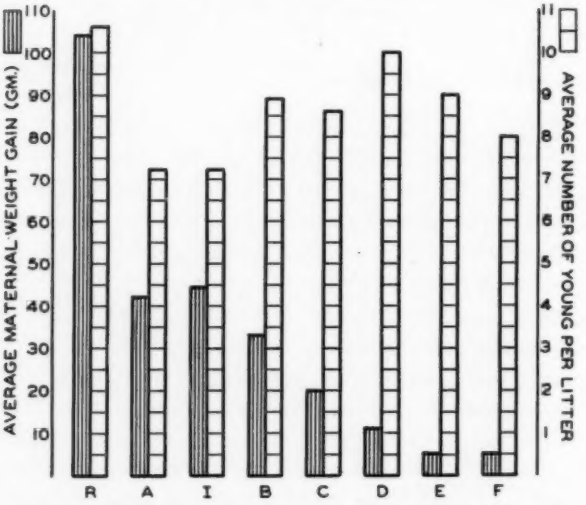

Fig. 1 Average maternal weight gain and average number of young per litter on control and experimental diets.

control diet, progressively decreasing to only 5% of the control weight gain when the supplement reached the 0.025% and 0.01% levels. It is interesting to note, however, that the average number of young per litter did not appear to be related to the tryptophan content of these rations. The lowest averages were obtained on the high tryptophan supplements, averages approximating those of the controls on the lower tryptophan supplements (fig. 1).

Dietary inadequacy was further apparent from the unkempt greasy coats of the animals and their general apathy. When the young were removed on the 22nd day of the gestation period, varying degrees of fat infiltration were grossly evident in the livers of all the mothers, including those on the 0.2% tryptophan supplement. This condition was found to be unrelieved by the addition to the ration of either a choline or methionine supplement.[5] Although neither Albanese and Buschke ('42) nor Totter and Day ('42) reported fatty livers when a similar diet was employed, Adamstone and Spector

TABLE 2

Incidence of congenital cataract

DIET	SUPPLEMENT	NO. OF LITTERS REPRESENTED	TOTAL NO. OF ANIMALS	NORMAL	ABNORMAL	
				no.	no.	%
A	0.2% tryptophan 10 mg % niacin	10	10	10	0	0
I	0.2% tryptophan	7	11	8	3	27
E and F	0.025–0.01% tryptophan 10 mg % niacin	9	11	4	7	64
R	control	9	11	10	1	9

('50), in a recent paper, have reported histological evidence of a heavy accumulation of fat in the livers of animals fed a tryptophan-deficient acid-hydrolyzed casein diet.

The incidence of congenital cataract as revealed by histological examination of the fetal eyes is shown in table 2. It will be noted that in the 10 animals representing 10 different litters from diet A, which contained the 0.2% tryptophan supplement plus 10 mg% niacin, not one case of cataract was found. However, in 11 animals from 7 litters on diet I, in

[5] Additional groups of animals were placed on a modification of diet A containing 0.2% choline and 0.6% methionine in an effort to determine whether or not these supplements would alleviate the fatty liver condition.

which the tryptophan supplement was at the same high level (0.2%) but to which no niacin was added, three animals had cataractous lenses. Since only one pair of litter mates from diet F was sectioned, these animals were grouped together

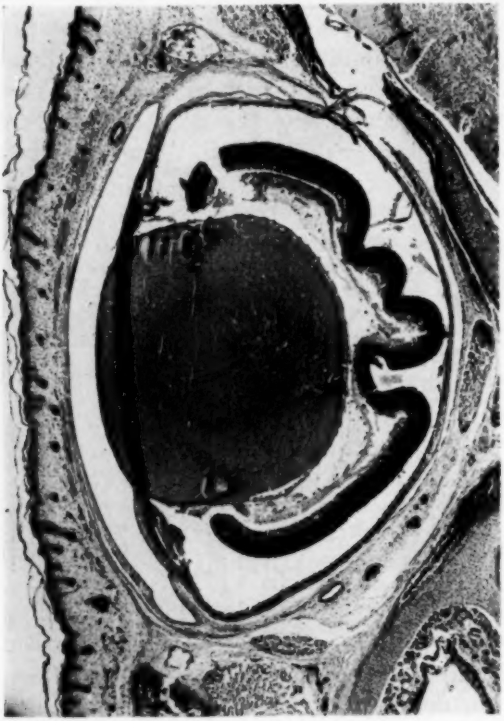

Fig. 2 Section of eye of rat fetus obtained from mother on diet A. In the crystalline lens of approximately normal shape and size, the fiber structure and the condition of the epithelium are essentially normal. The cracks are artifacts.

with those from diet E, giving a total of 11 animals representing 9 litters. At these low levels of tryptophan supplementation, notwithstanding the addition of niacin, 7 fetuses had cataractous lenses. These findings indicate that a 10 mg% niacin supplement at these low levels of trytophan intake

did not prevent cataract, nor was the 0.2% tryptophan level without additional niacin sufficiently high to permit an adequate supply of both tryptophan and niacin. However, the

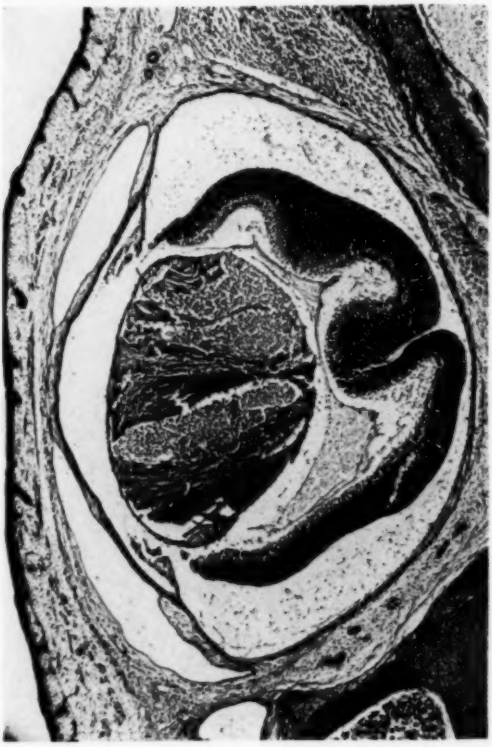

Fig. 3 Section of eye of rat fetus obtained from mother on diet I. In the crystalline lens, somewhat smaller than that of the control animals, zones of complete breakdown of the lens fibers are interposed between equatorial and axial areas of fairly well-preserved fiber structure.

addition of 10 mg% of preformed niacin at the 0.2% tryptophan level prevented cataract formation, although this supplement was not adequate for the restoration of normal liver function and normal growth in the maternal organism. It

is unlikely that the cataractous lenses in the animals from mothers on diets E and F were due to any single deficiency, since the extremely low food consumption on these diets was

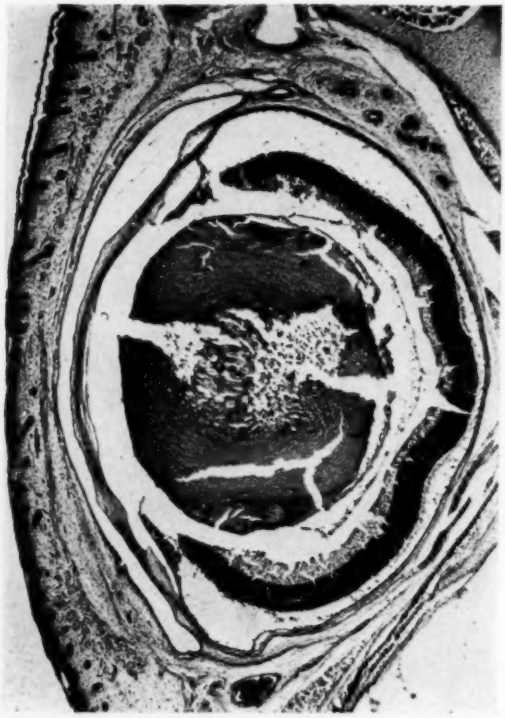

Fig. 4 Section of eye of rat fetus obtained from mother on diet E. In the crystalline lens of approximately normal shape and size the peripheral layers show fairly normal fiber structure. In the more central layers, varying degrees of disruption and actual breakdown are present.

undoubtedly responsible for multiple deficiencies, thus complicating the picture.

The control series, diet R, was characterized by normal lenses except for one animal which showed cataract formation. This cataract, however, was histologically of a very different

type than those observed in fetuses from animals on the experimental diets and was probably unrelated to a dietary deficiency, since cataracts of this type are known to occur in laboratory colonies of albino rats. Therefore, this was

Fig. 5 Section of eye of rat fetus obtained from mother on diet R. In the crystalline lens of approximately normal shape and size the fiber structure and the condition of the epithelium are essentially normal. The cracks are artifacts.

adjudged to be of no significance and unrelated to the factors examined in this study.[6] Photomicrographs of representative sections of the eyes of fetuses born of mothers on diets A, I, E and R are shown in the accompanying figures.

[6] Personal communication from Peter C. Kronfeld, M.D.

It appears that although the daily tryptophan requirement of the growing rat is thought to be 0.2% (Rose, '37), this level is not sufficiently high for the pregnancy period, as is evidenced by the findings cited. It is to be expected that the needs of the maternal organism during the gestation period would be higher than for growth or for the maintenance of the adult animal. Therefore it might be conceded that the need for tryptophan during the pregnancy period could be greater than the 0.2% which was added to the basal diet. However, the suggestion that the rat has no need for dietary niacin, since this vitamin can be made available by the metabolic conversion of tryptophan, leads to consideration of whether additional tryptophan is needed for the role it plays in protein synthesis or for conversion to niacin. The evidence of deficiencies in the maternal animals observed in this study, as well as the occurrence of congenital cataracts at the 0.2% tryptophan level (diet I) and their absence when this level of tryptophan was supplemented with 10 mg% niacin (diet A), are interpreted to be an expression of niacin limitation,[7] either in the form of its precursor, tryptophan, or the vitamin itself. This interpretation receives support in Hundley's ('47) finding that depressed growth and tissue deprivation of niacin occur when growing rats are maintained on a diet in which tryptophan is the chief amino acid limiting growth. Restoration of tissue levels and of growth occur with the addition of either tryptophan or niacin, the niacin levels in the liver returning to normal more slowly than in other tissues.

Cataracts may develop either because of impaired synthesis of lens protein or the degeneration of the lens fibers which have previously developed normally. Whereas it has been suggested by Albanese and Buschke ('42), Totter and Day

[7] An approximation of the daily niacin intake of these animals may be based on food intake records of pregnant animals in a subsequent study in which the same diets were employed and in which the average daily food consumption on diet R was 23 gm; on diet A, 9.7 gm, and on diet I, 9.7 gm. The niacin content of 23 gm of chow, according to the commercial analysis, is 3.13 mg. Assuming a 1% conversion of tryptophan to niacin, the amount of niacin received daily by animals on diets A and I was 1.31 and 0.34 mg, respectively.

('41, '42), Schaeffer and Geiger ('47) and Schaeffer and Murray ('50) that cataract in tryptophan deficiency is due to disturbed protein synthesis, interpretation of the findings in this study indicates that the cataract of tryptophan deficiency may well be due to a metabolic disturbance in the lens, with consequent degeneration of the lens fibers. This metabolic disturbance, due to a deficiency of niacin — the critical level being at some point between the amount received in diet I and that received in diet A — could, by curtailing the formation of enzymes of which niacin is the prosthetic group, interfere with the intermediary metabolism of carbohydrate. Since biochemical investigations of lens metabolism indicate that in this tissue carbohydrate is metabolized exclusively, it is evident that niacin deficiency could lead to a derangement in the normal process, with the resulting accumulation of intermediary metabolites. This interference with the respiration of the lens could then lead to the subsequent degeneration of lens fibers and to the condition known as cataract. It is known that a variety of other factors interfering with carbohydrate metabolism in the lens can lead to cataract; e.g., riboflavin deficiency, xylose and galatose feeding, naphthalene poisoning and diabetes. It appears justifiable to assume, therefore, that niacin deficiency could lead to a comparable block in the normal metabolism of carbohydrate in the lens, with subsequent interference in cell respiration and the degeneration of lens fibers.

The reports of Totter and Day ('41, '42) describing cataract as a result of tryptophan deficiency imply that possibly some of the accompanying changes in their animals were not due solely to a deficiency of this amino acid. It was suggested by Albanese and Buschke ('42) that the variety of characteristic lesions in tryptophan deficiency would support the view that tryptophan has specific functions to perform in addition to serving as a building block for body proteins. Further evidence concerning the dual role of tryptophan in metabolism has been supplied by Geiger, Hagerty and Gatchell ('49). Schaeffer and Geiger ('47) had previously reported that

tryptophan fed as a delayed supplement to rats on a tryptophan-free diet neither promoted growth nor prevented cataract formation. However, when they employed a basal diet which contained a sufficient amount of tryptophan for maintenance (0.1% in the form of casein) they found that a delayed supplement of either tryptophan or niacin promoted growth as well as when fed simultaneously with the basal diet. They also showed that this delayed supplement of tryptophan resulted in a considerable increase in niacin excretion. Although they attribute the positive results of their experiment to the correction by tryptophan of the "harmful effect of the imbalanced amino acid mixture," these results could be interpreted to show the presence of a niacin deficiency correctable by either niacin or tryptophan.

In reporting on tryptophan-deficiency cataract, Buschke ('43) noted that lesions developed in the lenses of his control animals. These rats received a diet (basal plus 0.225% tryptophan) similar in composition to the one employed in the study by Totter and Day ('41) and to diet I in the present study. These lesions, observed by slit lamp examination, were attributed to some exogenous damage associated with the tryptophan control diet. Buschke also suggested that the lens-damaging factor or factors intrinsic in the tryptophan control diet in some way favored the manifestations of damage to the lens encountered in the rats on the tryptophan-deficient diet. The data in the present study indicate that this, indeed, is the case, and that the "lens-damaging factor" which Buschke suggested was a deficiency of available niacin. This is evident from the appearance of cataract in the young born of mothers receiving a diet essentially the same as Buschke's tryptophan control diet (diet I), and the absence of cataract when the mothers received this diet with the niacin supplement (diet A).

It appears, therefore, that a 0.2% tryptophan supplement to an acid-hydrolyzed casein diet is insufficient for both the tryptophan and niacin requirements of the pregnant rat. The addition to such a diet of 10 mg% of preformed niacin per-

mitted the resumption of normal metabolism in the fetal lens, but this level did not permit normal growth and proper liver function in the maternal organism. It would seem that the resumption of normal metabolism in the liver and of normal growth would require either more tryptophan, more niacin, or possibly additional amounts of some other factor or factors which may have been lacking in the diet employed. Further investigation of this problem is being undertaken.

SUMMARY

Pregnant rats fed an acid-hydrolyzed casein diet supplemented with 0.2% tryptophan produced young with congenital cataracts. When this diet was further supplemented with 10 mg% niacin, the eyes of the young were normal. This combination, however, was insufficient for normal weight gain and for prevention of fat accumulation in the livers of the mothers.

The 10 mg% niacin supplement was without effect in protecting the fetal lens when the tryptophan content of the maternal diet was reduced to 0.025%.

The possibility that the cataracts produced by tryptophan-deficient diets of the type used in this study may be due to the limited amount of tryptophan available for conversion to niacin is discussed.

ACKNOWLEDGMENTS

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THE EFFECT OF CANNING PROCEDURES ON THE NUTRITIVE VALUE OF THE PROTEIN IN PEAS[1]

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INTRODUCTION

Dried peas *(Pisum sativum)* are widely used as a source of protein in low cost diets. They are also valuable for animal feeding, because the percentage of protein is about twice that in cereal grains. Johns and Finks ('21), Woods, Beeson and Bolin ('43), and Peterson, Lampman, Bolin and Stamberg ('44) have shown that methionine is the limiting amino acid in peas.

Woods, Beeson and Bolin ('43) reported that heat treatment impairs the protein efficiency of peas. When peas were autoclaved for one and one-half hours at 255°F. and fed to rats, the protein efficiency was about half that of the raw pea. Murray ('48) found that a canning procedure in which peas were autoclaved for 40 minutes at 240°F. lowered the protein efficiency significantly. Evans and St. John ('48) showed that the liberation of amino nitrogen by proteolytic enzyme digestion was decreased following autoclaving at 130°C. for 60 minutes; 24% of the cystine, 25% of the lysine, and none of the methionine were destroyed by this process.

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The reduction in protein efficiency may be a result of a combination involving certain amino acids of the pea protein and a reducing sugar in a linkage stable to enzyme action. Stevens and McGinnis ('47) reported that lysine autoclaved with cerelose was unavailable for chick growth, whereas heating lysine alone had no effect on its supplementary value. Evans and Butts ('49b) observed three types of inactivation of amino acids in soybean protein as a result of prolonged autoclaving with sucrose. The amino acids with free amino groups react with sucrose to destroy the amino acid. Lysine, aspartic, and glutamic acids combine with some constituents of the protein, probably the free carboxyl, and with the free amino groups to form enzyme-resistant linkages. Protein-bound methionine, cystine and histidine form linkages with sucrose resistant to enzymatic hydrolysis *in vitro*. Patton, Hill and Foreman ('48) refluxed vitamin-test casein with glucose for 24 hours. Subsequent amino acid assays indicated that of the 9 amino acids assayed only lysine, arginine and tryptophan were appreciably inactivated. Stewart and Kline ('48) found that reactions between glucose and egg white protein were responsible for color, fluorescence, and solubility changes in dried albumin during storage. When amino acids were added, they acted preferentially with the glucose and slowed down deterioration.

Fraenkel-Conrat and Olcott ('48a, b) reported that stable cross-linking bridges between amino groups on the one hand and amide, guanidyl, phenol, imidazole, or indole groups on the other, through formaldehyde, occur under varying conditions of pH and temperature. This may be the mechanism for the reaction between amino acids and reducing sugars in peas.

The digestibility of soybean oil meal was decreased for the chick following autoclaving at 130°C. for 30 minutes (Evans, McGinnis and St. John, '47). Mayfield and Hedrick ('49) reported that canning beef lowered the digestibility of its protein for rats. Mitchell, Hamilton and Beadles ('50), using rats, found that the lowered biological value of the proteins

of heat-treated peanut meal, sunflower seed meal, and cottonseed flour was due to decreased digestibility.

The purpose of the present study was to investigate the effect of canning procedures on the efficiency and digestibility of the protein of peas by the use of animal assays, chemical methods for the determination of alpha-amino nitrogen and reducing sugars, and a microbiological measurement of methionine.

PART I. PROTEIN EFFICIENCY

Woods, Beeson and Bolin ('43) showed that the methionine content of the protein of peas is a limiting factor for rat growth. When they supplemented diets containing 10% raw pea protein with 0.3% methionine, a protein efficiency higher than that obtained with casein was secured. Lysine was also used as a supplement in raw pea rations, since the content of this amino acid is lower in peas than in animal proteins such as egg and milk. Evans and Butts ('49a) found that when soybean oil meal was autoclaved for 4 hours, the heat treatment inactivated some of the lysine content. Canned pea diets were therefore supplemented with lysine, to determine whether canning procedures resulted in a linkage between the reducing sugar and lysine content of the pea. Block and Mitchell ('46–'47) reported that 5% of pea protein is lysine. By supplementing the pea diet with 0.5% lysine, a total content of 1.0 gm per 100 gm of diet was secured.

Materials and methods

Preparation of the peas. Three samples of mature Alaska field peas were used. Two of the samples were obtained from a local pea processing plant, and the third from the Department of Agronomy on this campus. Preparation included soaking overnight, parboiling in the same water for 5 minutes, canning, autoclaving at 10 lb. pressure for 40 minutes, drying the contents of the can in a dehydrator at 135°F. for 24 hours, and grinding to a flour. To discover whether pH had any effect on the amino acid-glucose reaction, two

lots of peas were canned at a pH of 4.5 and 8.5 respectively, using acetate buffers in place of water. During the canning process, when these buffers were used, a change in pH occurred, resulting in final figures of 4.9 and 6.1, respectively; the pH of the water control was 6.1. The dried products were analyzed for moisture and nitrogen, using the Kjeldahl-Gunning-Arnold method (Association of Official Agricultural Chemists, '45).

Animal assay. The protein efficiency of the peas was determined by the rat growth method. Young rats weighing 40 to 60 gm were selected for experiment. They were housed in individual cages, weighed three times weekly, and fed ad libitum. Trials were run for 5 weeks. Each rat received as a supplement three times weekly a vitamin solution containing thiamine hydrochloride 50 μg, riboflavin 50 μg, pyridoxine 50 μg, calcium pantothenate 250 μg, nicotinic acid 580 μg, inositol 130 μg, choline chloride 20 mg, *p*-aminobenzoic acid 250 μg, vitamin A 330 I.U., and vitamin D 33 I.U. Diets were prepared from the raw and processed peas; the quantity of each sample required to furnish a protein level ($N \times 6.25$) of 10% was used in making up the diets (table 1). When amino acids were used as supplements, they were thoroughly mixed with the diet.

The apparent digestibility of raw and canned peas was determined. Animals were housed in individual metabolism cages and fed the same diet as that used in the protein efficiency study. Feces were collected every other day. Since the rats were placed on this experiment immediately after completing the efficiency study, no adjustment period was necessary. Collections were made for a period of 6 days. The feces were dried, ground, and thoroughly mixed. Nitrogen was determined by the Kjeldahl-Gunning-Arnold method. The following formula was used to calculate the apparent digestibility:

$$\frac{\text{Nitrogen intake in diet} - \text{nitrogen output in feces} \times 100}{\text{Nitrogen intake in diet}}$$

Results and discussion

The results of the growth studies are summarized in table 2.

Canning in water alone or in a buffer of pH 8.5 did not cause a significant change in the protein efficiency of any sample. Canning in a buffer of pH 4.5, however, lowered this value. Rats on diets composed of peas canned in both buffers

TABLE 1

Composition of diets

SAMPLE	TREATMENT	PEA[1] FLOUR	STARCH	HYDROGENATED FAT	HUBBELL SALTS	AMINO ACIDS	
						DL-Methionine	L-lysine
		%	%	%	%	%	%
I	Raw	36.5	56.0	5	2.5	..	..
	Canned	40.3	52.2	5	2.5	..	..
	Canned at pH 4.5	38.5	54.0	5	2.5	..	..
	Canned at pH 8.5	45.0	47.5	5	2.5	..	..
II	Raw	38.6	53.9	5	2.5	..	..
	Canned	40.4	52.1	5	2.5	..	..
	Raw + methionine	36.8	55.5	5	2.5	0.15	..
	Raw + lysine	36.8	55.2	5	2.5	..	0.50
	Canned + lysine	36.8	55.2	5	2.5	..	0.50
III	Raw	39.1	53.4	5	2.5	..	..
	Canned	38.8	53.7	5	2.5	..	..

[1] All diets contained pea flour sufficient to provide 10% protein.

and on diets containing canned samples I and II showed significantly lower weight gains than rats consuming diets made from the corresponding raw peas.

Murray ('48) obtained higher values for the protein efficiency of raw peas than those secured in this study. There is apparently some variability in the values obtained with different samples of peas. There was a significant difference in this work between the protein efficiency in samples I and II on the one hand and sample III on the other. Work in this

TABLE 2

Growth response on diets used to determine protein efficiency of pea diets

SAMPLE	DIETS COMPARED	AVE. FOOD INTAKE/DAY		AVERAGE TOTAL GAIN [1]			PROTEIN EFFICIENCY [1]		
		(a)	(b)	(a)	(b)	"t" [2]	(a)	(b)	"t" [2]
I	Raw (a) vs. canned (b)	8.2	8.0	33.8 ± 1.4	29.3 ± 1.4	2.25 [3]	1.04 ± 0.04	1.05 ± 0.05	0.17
	Raw (a) vs. canned at pH 4.5 (b)	8.2	8.5	33.8 ± 1.4	27.8 ± 0.6	2.97 [4]	1.04 ± 0.04	0.84 ± 0.12	2.12 [3]
	Raw (a) vs. canned at pH 8.5 (b)	8.2	8.6	33.8 ± 1.4	29.1 ± 1.3	2.15 [3]	1.04 ± 0.04	1.07 ± 0.05	0.49
II	Raw (a) vs. canned (b)	8.6	8.6	31.8 ± 1.6	31.4 ± 0.01	0.19	0.97 ± 0.04	1.01 ± 0.04	0.67
	Raw (a) vs. raw + methionine (b)	8.6	11.9	31.8 ± 1.6	85.9 ± 3.3	5.53 [4]	0.97 ± 0.04	2.03 ± 0.06	5.83 [4]
	Raw (a) vs. raw + lysine (b)	8.6	8.8	31.8 ± 1.6	36.5 ± 1.7	1.90	0.97 ± 0.04	1.16 ± 0.04	3.17 [4]
	Canned (a) vs. canned + lysine (b)	8.6	8.6	31.4 ± 0.01	24.8 ± 1.0	4.10 [4]	1.01 ± 0.04	0.82 ± 0.04	3.45 [4]
III	Raw (a) vs. canned (b)	9.9	8.6	40.2 ± 3.2	36.8 ± 1.8	2.97 [4]	1.20 ± 0.07	1.13 ± 0.03	0.91
	Raw I (a) vs. raw II (b)	8.2	8.6	33.8 ± 1.4	31.8 ± 1.6	1.00	1.04 ± 0.04	0.97 ± 0.04	1.40
	Raw I (a) vs. raw III (b)	8.2	9.9	33.8 ± 1.4	40.2 ± 3.2	2.18 [3]	1.04 ± 0.04	1.20 ± 0.07	2.25 [3]

[1] Standard error included.

[2] Calculated from Fisher's formula (Snedecor, '46).

[3] Significant at the 5% level.

[4] Significant at the 1% level.

laboratory with several samples of raw peas grown under different conditions has shown some fluctuation in protein efficiency (unpublished data).

Rats eating approximately the same quantities of diet grew faster when methionine supplemented the ration than when the raw pea diet was fed alone; the efficiency of the protein of the ration was therefore significantly increased by this supplement. Supplementing with lysine significantly increased the protein efficiency but not the weight gain. However, when lysine was added to canned peas, significantly lowered values resulted for both protein efficiency and weight gain. A possible explanation for this finding may be that, through cross-linking with reducing sugars of the pea, some amino

TABLE 3

Results of digestibility study of pea diets

SAMPLE	TREATMENT	NUMBER OF ANIMALS	AVERAGE NITROGEN INTAKE PER 6 DAYS	AVERAGE NITROGEN OUTPUT PER 6 DAYS	APPARENT DIGESTIBILITY
			gm	*gm*	%
II	Raw	6	0.594 ± 0.008	0.143 ± 0.010	76.0
II	Canned	5	0.681 ± 0.018	0.200 ± 0.007	70.7

acids were made unavailable to the rat, and subsequent lysine supplementation may have disturbed the amino acid balance of the diet. Roth and Allison ('50) reported that when excess methionine was fed to rats, a marked loss in weight occurred and that glycine would in part counteract this effect.

Results of the digestibility study are summarized in table 3. The "t" value (Snedecor, '46) for the digestibility of the raw vs. canned peas was 2.59, which indicates that canning lowered the digestibility somewhat.

PART II. CHEMICAL AND MICROBIOLOGICAL STUDIES

To observe whether the sugar present in peas may combine with free amino acid groups of the protein during the canning process, the reducing sugar and alpha amino nitrogen content

were determined before and after canning. Methionine was assayed microbiologically in the raw and canned peas.

Materials and methods

The reducing sugar content was determined by the method of Hassid ('36). The alpha amino nitrogen was measured using the copper method of Pope and Stevens ('39) on acid and enzyme hydrolysates. Ten per cent suspensions of the pea flour in 2N hydrochloric acid were autoclaved for three hours at 250°F. for the acid digestion. The enzymatic hydrolysis was carried out by the procedure of Evans ('46) using pepsin, trypsin, and erepsin. Methionine was assayed microbiologically on enzymatic digests of the peas, using the organism *Leuconostoc mesenteroides P-60*. The preparation of the hydrolysates for this assay was the same as that of the enzymatic digestion used for the amino nitrogen determination. Henderson and Snell's ('48) medium, with modifications by Chang and Murray ('49), was employed.

Results and discussion

The results of the reducing sugar, alpha amino nitrogen and methionine determinations are given in table 4.

Enzyme hydrolysis liberated more alpha amino nitrogen than acid hydrolysis in the majority of the samples.

The reducing sugar and alpha amino nitrogen contents of the peas were decreased by canning both in water and in buffers of pH 4.5 and 8.5. This seems to indicate a combination between the sugar and alpha amino groups unhydrolyzed by either acid or enzymes. Since the protein efficiency of the canned peas did not show a reduction, it appears that the combination formed involved none of the essential amino acids, or that the digestive enzymes of the rat were able to hydrolyze the linkage. The methionine determinations, however, show that the content of this amino acid is somewhat lower in the canned peas than in the raw. Some methionine presumably has combined with the reducing sugar. Graham,

TABLE 4

Reducing sugar, amino nitrogen and methionine content of peas

SAMPLE	TREATMENT	REDUCING SUGAR mg %	DIFFERENCE	AMINO NITROGEN % OF TOTAL NITROGEN				METHIONINE % OF PROTEIN	DIFFERENCE	METHIONINE GRAMS PER 100 GM DIET	% OF ROSE'S (48)[1] REQUIREMENT (0.8 GM PER 100 GM DIET)
				Acid hydrolysis	Difference	Enzyme hydrolysis	Difference				
I	Raw	103.5	...	40.4	...	39.0	..	1.62	...	0.172	21.5
	Canned	73.0	30.5	25.2	15.2	31.4	7.6	1.41	0.21	0.138	17.2
	Canned pH 4.5	77.2	26.3	20.7	19.7	32.9	6.1	...	...	....	...
	Canned pH 8.5	59.4	44.1	22.7	17.7	36.4	2.6	...	...	....	...
II	Raw	155.0	...	38.8	...	39.5	..	1.95	...	0.213	26.6
	Canned	89.1	65.9	25.8	13.0	28.5	11.0	1.40	0.55	0.144	18.0
III	Raw	157.4	...	53.7	...	41.7	...	2.15	...	0.218	27.2
	Canned	78.9	78.5	51.2	2.5	26.4	15.3	1.90	0.25	0.181	22.5
	Average		49.1		13.6		8.5		0.33		

[1] Rose, W. C., M. J. Oesterling and M. Womack. Comparative growth on diets containing 10 and 19 amino acids, with further observations upon the role of glutamic and aspartic acids. J. Biol. Chem., *176:* 753–762 (1948) .

Hsu and McGinnis ('49) found that methionine when autoclaved with glucose or lactose was unavailable for chick growth. In the present study the lowered methionine values did not apparently alter the protein efficiency. Values for the protein efficiency were higher for the third sample of raw peas than for either of the others. This cannot be explained in terms of the methionine content, since samples II and III contained more of this amino acid than sample I.

SUMMARY

1. Three samples of Alaska field peas studied showed that canning procedures did not affect the protein efficiency. Rats on diets containing canned peas gained less weight than animals on the corresponding raw pea diets.

2. When the peas were canned at a pH of 4.5, there was a significant decrease in the protein efficiency; when the canning was done at a pH of 8.5, the weight gain was significantly decreased.

3. Supplementing raw peas with methionine substantially raised the protein efficiency. When lysine was used as a supplement for raw peas, a significant increase in protein efficiency resulted; but when it was used as a supplement for canned peas a significant decrease became apparent.

4. A digestibility study showed that raw peas are somewhat more digestible than canned.

5. The reducing sugar, alpha amino nitrogen, and methionine contents of the peas were decreased by canning procedures.

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THE EFFECT OF HEAT TREATMENT ON THE NUTRITIVE VALUE OF MILK PROTEINS

III. THE EFFECT OF HEAT ON CASEIN, LACTALBUMIN, AND THEIR LACTOSE-INDUCED DERIVATIVES, WITH SPECIAL REFERENCE TO DIGESTIBILITY AND RATE OF RELEASE OF LYSINE, METHIONINE AND TRYPTOPHAN

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FOUR FIGURES

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It has been demonstrated in paper I of this series (Cook, Morgan, Weast and Parker, '51) that heat used in the sterilization of evaporated and powdered milks decreased the protein efficiency in proportion to the degree and length of time of heating. In paper II (Cook, Morgan, Singer and Parker, '51) heat was found to cause damage to the nutritive value of lactose-free casein and lactalbumin, and the damage was intensified if the proteins were combined with lactose before heating.

Rose ('38) demonstrated that the nutritive quality of proteins was dependent upon the biological availability as well as the content of their constituent amino acids. Melnick, Oser and Weiss ('46) have suggested that differences in the biological value of proteins may be due to differences in the rate of enzymatic release of their respective amino acids during digestion. Several investigators have found that heated casein is less readily digested than the raw material (Block, Jones and Gersdorf, '34; Pader, Melnick and Oser, '48).

The importance of the browning reaction between reducing sugars and proteins is becoming increasingly evident, both as a nutritional and chemical problem. Henry, Kon, Lea and White ('48) observed that storage of skim milk powders under varying conditions of temperature and humidity led to browning and a resulting decrease in palatability, solubility, free lactose, pH, free amino nitrogen, and available lysine, histidine, arginine, and methionine. Other workers (Patton, Hill and Foreman, '48; McInroy, Murer and Thiessen, '49) have reported an intensification of these reactions upon autoclaving casein with glucose. Mader, Schroeder and Smith ('49) found, in a nitrogen balance study with dogs, that the digestibility and biological value of a lactalbumin [1] which contains lactose were less affected by heat after removal of the dialyzable constituents.

Mohammed, Fraenkel-Conrat and Olcott ('49), in studying the nature of the browning reaction between crystalline bovine serum albumin and glucose solutions, found the resulting brown material to contain about 20% of bound glucose and to have a different isoelectric point, an increased molecular weight, and decreased digestibility by trypsin. Heat treatment of this new product caused further decrease in digestibility. Acetylated bovine serum albumin did not brown under their conditions, thus indicating the necessity of free amino groups for the browning reaction.

Lea and Hannan ('50), in studying the reaction of sodium caseinate and glucose, were able to distinguish three stages of interaction: (1) reaction of free amino groups, primarily the α-amino groups of lysine, with glucose in a ratio of 1:1, resulting in a white product of unimpaired solubility; (2) formation of a browned product accounting for more carbohydrate than amino moieties and involving also arginine, histidine, methionine, and tryptophan; and (3) production of a darker colored product of increased molecular weight.

[1] Borden's Labco (15–42).

EXPERIMENTAL METHODS

In the experiments to be reported, the following proteins were used: raw commercial casein and three types of lactalbumin; (a) commercial, salt precipitated, lactose-free, referred to as A(soluble);[2] (b) laboratory prepared, ethanol-precipitated, lactose-free; and (c) a commercial lactose-containing sample, B(15–42).[3] These preparations have been described in paper II of this series. Each of these proteins was used unheated and combined with lactose under the mild and severe conditions, and each of the resulting products was tested before and after heat treatment.

The mild method of lactose treatment was carried out at 52°C. in an air oven for 24 hours. The ratios of protein to lactose used were 1:0.3; 1:0.4; 1:2; and 3.5:5.0 in solutions containing 2 ml of 0.2 M or 3 M phosphate buffer (pH 7.6) and 6 ml of water per gram of protein. The solutions were dialyzed against running tap water and then distilled water, concentrated in an air current, and lyophilized.

The severe lactose treatment was carried out essentially according to the method of Patton, Hill and Foreman ('48), using 100 ml of 5% lactose instead of glucose solution, and 5 gm of protein, refluxing at 95°C. for 24 hours, dialyzing, concentrating in an air current, and lyophilizing.

Heating of the proteins and their lactose derivatives was carried out in shallow layers in an air oven at 118°, 140°, and 150°C. for 30 or 70 minutes.

The acetylation of the proteins was carried out by the method of Olcott and Fraenkel-Conrat ('47), using acetic anhydride at 0°C., which is specific for blocking only amino groups.

The α-amino nitrogen was determined either manometrically for three minutes by the method of Van Slyke ('29), or colorimetrically by the ninhydrin method of Harding and MacLean ('15), using the Beckman spectrophotometer, and

[2] We are indebted to the Avoset Company of San Francisco for generously supplying this lactalbumin.

[3] See footnote 1, page 218.

absorbing at wave-length 572 mμ with a slit-width of 0.135 mm. These analyses were made upon enzyme hydrolysates of the various protein preparations and found to check each other whenever both methods were used on the same sample.

The total nitrogen was determined by the micro-Kjeldahl method, using selenium pellets and 30% hydrogen peroxide as catalysts.

The lactose uptake by these proteins was determined by the difference in the nitrogen content before and after lactose treatment. It is impossible to determine lactose per se after it has reacted with protein, since it has lost its reducing quality in condensing with the protein. It is hoped in the future to check the lactose uptake of the protein by use of radioactive lactose and to measure the specific activity of the condensation product.

Basic groups of the intact proteins were determined by the dye-binding method of Fraenkel-Conrat and Cooper ('44) on 5-mg samples at three levels.

The amino acids lysine, methionine, and tryptophan were determined microbiologically on both acid and enzyme hydrolysates of the protein preparations. The organisms *L. mesenteroides* P-60 for lysine and methionine, and *L. arabinosus* for tryptophan were used with complete basal media.[4] They were incubated at 37°C. for 72 hours. Duplicate or triplicate analyses were made for each value in each experiment.

The acid hydrolysates, on which the total amino acids were determined, were prepared by autoclaving 100-mg samples of protein with 1 ml of 10% HCl in sealed tubes for 10 hours at 15 lb. pressure, filtering, and making up to 100 ml after adjusting the pH to 6.8. They were stored frozen until the analyses were made.

The enzyme hydrolysates, on which the biologically available amino acids were determined, were prepared as follows: 100 mg protein and 0.6 mg crystalline pepsin in 2 ml of 0.1 N H_2SO_4 were incubated at 37°C. for three days; the pH of the solution was adjusted to 7.6 to 8.0 with NaOH,

[4] Obtained from the H. M. Chemical Company, Los Angeles, California.

0.5 mg crystalline trypsin and 0.2 mg crystalline chymotrypsin were added, and the solution was incubated three more days; finally, 0.1 ml of a suspension of carboxypeptidase [5] (activity unknown) was added, and the mixture was incubated another 24 hours; the pH was adjusted to 6.8, and the volume made up to 100 ml. These preparations were stored frozen.

The rate of enzyme hydrolysis of the various proteins and their derivatives was determined as follows: 100 mg protein, 5 mg Pangestin (Difco), 20 ml phosphate buffer (pH 7.6), and water to make 100 ml final volume were incubated at 37°C. Aliquots were removed at intervals of one to 72 hours, and the degree of hydrolysis was followed by the determination of α-amino nitrogen and the release of lysine, methionine, and tryptophan by microbiological assay.

Total tryptophan was determined chemically by the method of Horn and Jones ('45) on enzyme hydrolysates.

Moisture was determined on 100- to 500-mg samples dried to constant weight at 105°C. in an air oven.

RESULTS AND DISCUSSION

Both casein and lactalbumin were found to combine chemically with lactose, the amount combined depending upon the experimental conditions and the nature of the protein (table 1). About twice as much lactose reacted with casein under the severe conditions as under the mild conditions. The lactalbumins seemed to become saturated with lactose under the mild conditions, and even when refluxed in aqueous solutions containing equal amounts of protein and lactose there was no additional uptake of lactose.

In a later experiment (table 2) designed to test the effect on the browning reaction of varying the concentration of the buffer and the lactose, only the mild conditions were used. The protein-lactose reaction was unaffected by the concentration of the phosphate buffer used (0.2 or 3.0 M). Variation in the ratio of protein to lactose from 1:0.3 to 1:2 led to an

[5] We are indebted to Dr. A. K. Balls of the Western Regional Research Laboratories, Albany, California, for the extract containing carboxypeptidase.

increased uptake of lactose in the lactalbumin but not in the casein. It should be noted, however, that a greater concentration of lactose, particularly in the ratio of protein to lactose found in milk (3.5 : 5.0), led to darker final products than did smaller concentrations of lactose, indicating more damage to the original protein by internal rearrangements, condensations, or cross-links. This hypothesis is further substantiated by a decrease in the free amino groups and basic groups of the intact protein and in the degree of digestion and liberation of lysine and methionine.

TABLE 1

The lactose uptake of casein and lactalbumins in two methods of lactose reaction and after acetylation

TREATMENT OF PROTEIN	PER CENT LACTOSE UPTAKE [1]		
	CASEIN	A (soluble)	Ethanol-precipitated
None	0	0	0
Mild lactose [2]	4–5	2–3	2–3
Severe lactose	7–8	2–3	2–3
Acetylated	0 [3]	..	0 [4]
Acetylated + lactose, mild	2–3	..	0

[1] Lactose uptake was determined by difference in the nitrogen content of the protein before and after reaction with lactose.

[2] Ratio of protein to lactose, 1 to 0.3.

[3] Acetate content, 6%. Determined by difference in the nitrogen content of the protein before and after reaction with acetic anhydride at 0°C.

[4] Acetate content, 3%.

In the earlier experiments it was noted that when the proteins were subjected to the lactose reaction at 52°C. for 24 hours there was, contrary to expectations, an increase in the free amino nitrogen (Van Slyke, '29) and basic groups of the intact protein, and occasionally also in the enzymatic release of amino acids. The only feasible explanation for such a finding, if the protein and lactose really combined, is that some hydrolysis of peptide bonds also occurred during the process. The results in table 2 confirm this supposition. When the two proteins were incubated in the buffer solutions with-

TABLE 2

The effect of varying the concentration of phosphate buffers (pH 7.6) and the ratio of protein to lactose on the amount of lactose combined and on certain chemical properties of casein and lactalbumin during mild lactose treatment [1]

PHOSPHATE BUFFER	RATIO OF PROTEIN TO LACTOSE	LACTOSE COMBINED	AMINO [2] NITROGEN	BASIC GROUPS 10^4 gm	AMINO [3] NITROGEN	LYSINE		METHIONINE		TRYPTOPHAN [5]		COLOR OF FINAL PRODUCT
						Method of hydrolysis [4]						
						Enzyme	Acid	Enzyme	Acid	m.b.	Chemical	
					Dialyzed A (soluble) lactalbumin							
None	No lactose	..	0.92	9.6	5.4	8.4	9.9	1.5	1.9	2.1	2.3	White
0.2 M	No lactose	..	1.45	10.0	7.3	9.8	..	1.8	..	2.3	2.4	White
0.2 M	1:0.3	2	1.11	10.2	6.6	8.1	..	1.7	..	2.2	2.3	White
3 M	No lactose	..	1.30	9.8	6.7	8.6	..	1.8	..	2.3	2.3	White
3 M	1:0.4	2	0.99	9.7	6.9	8.4	..	1.7	..	2.2	2.2	Cream
3 M	1:2	5	0.79	8.7	4.8	7.2	9.9	1.1	1.9	1.9	2.2	Tan
3 M	3.5:5	5	0.88	8.5	4.8	7.1	8.8	1.2	1.9	2.1	2.2	Light tan
					Dialyzed casein							
None	No lactose	..	0.78	8.3	5.0	6.7	7.9	1.4	2.3	1.3	1.4	Cream
0.2 M	No lactose	..	1.21	8.1	5.0	6.5	..	1.5	..	1.5	1.5	Cream
0.2 M	1:0.3	5	0.86	8.1	4.8	6.2	..	1.5	..	1.1	1.2	Tan
3 M	No lactose	..	1.06	7.0	5.8	6.4	..	1.9	..	1.3	1.3	Cream
3 M	1:0.4	5	0.83	7.7	4.9	5.4	..	1.5	..	1.2	1.3	Tan
3 M	3.5:5.0	4	0.60	6.5	4.4	5.2	7.2	1.2	2.1	1.3	1.4	Brown

[1] Values are given in per cent of the protein corrected to the nitrogen of the original sample, which was 13.7% for lactalbumin and 14.6% for casein.

[2] Determined by the Van Slyke method on the intact protein.

[3] Alpha-amino nitrogen determined by the ninhydrin method in enzyme hydrolysates as a measure of the amount of digestion.

[4] Pangestin was used for enzyme hydrolysis.

[5] Tryptophan was measured microbiologically (m.b.) and chemically in enzyme hydrolysates of the proteins.

out lactose, the free amino nitrogen was increased in both cases; and when the proteins were incubated with lactose in the buffered solutions there was less free amino nitrogen than in the buffered controls.

The effect of heat of varying degrees and of other conditions on the digestibility of the lactose-free and lactose-containing proteins, as measured by the release in α-amino nitrogen and available lysine, methionine, and tryptophan in enzyme hydrolysates (72 hours digestion), is shown for A(soluble) and ethanol-precipitated lactalbumins in figure 1 and for casein in figure 2. Only a slight decrease in digestibility and in biologically available amino acids was found in the A(soluble) lactalbumin as a result of dry heat, as compared with a somewhat greater decrease in casein and ethanol-precipitated lactalbumin. The latter protein was probably slightly denatured in the course of preparation because of inadequate cold room facilities in which to carry out the precipitation. Also, because of the yellowish color of commercially prepared casein as compared with the white color of the laboratory prepared casein, it is not known how much primary denaturation of the casein may have been incurred in its commercial preparation. Similarly, mild lactose treatment of A(soluble) lactalbumin and subsequent heat treatment were less deleterious to this protein than similar treatments were to casein and ethanol-precipitated lactalbumin, both of which showed marked decreases in available lysine and methionine. Severe lactose treatment caused not only a decrease in *in vitro* digestibility and release of amino acids but also some actual destruction of lysine and, in some instances, of methionine and tryptophan. Further heat treatment of these proteins refluxed in a lactose solution intensified these effects.

B(15–42) lactalbumin (fig. 2), presumably because of its lactose content and preparative heating (this preparation was light tan in color in contrast to A(soluble) lactalbumin which was white), responded to dry heat in much the same manner as did the other proteins which had been refluxed in a lactose solution prior to dry heating.

Rat growth studies, reported by Cook, Morgan, Singer and Parker ('51) in paper II of this series, showed a decrease in the protein value of mild lactose-treated casein in proportion to the decrease observed by microbiological assay in

TABLE 3

Effect of acetylation, lactose and heat [1] on casein and ethanol-precipitated lactalbumin

KIND OF PROTEIN AND TREATMENT	AMOUNTS IN RAW MATERIAL [2] AND DECREASE [3] AS RESULT OF TREATMENT			
	α-amino nitrogen [4]	Lysine	Methionine	Tryptophan
	%	%	%	%
Casein:				
None	5.2	4.5	1.9	1.3
Acetylated [5]	5.2	0.8 (82)	1.7 (10)	1.4
Same + heat	5.2	0.4 (91)	1.4 (26)	1.3
Acetylated + lactose [6]	5.1	0.7 (84)	1.5 (21)	1.4
Same + heat	5.6	0.4 (91)	1.5 (21)	1.7
Lactalbumin:				
None	5.7	5.7	1.4	2.6
Acetylated [7]	5.0 (12)	2.1 (63)	1.3 (7)	2.4 (8)
Same + heat	4.9 (14)	1.3 (77)	1.3 (7)	2.4 (8)
Acetylated + lactose [6]	5.3 (7)	2.5 (52)	1.5	2.5 (3)
Same + heat	5.3 (7)	1.6 (72)	1.4	2.5 (3)

[1] Heated at 150°C. for 70 minutes.

[2] All values were determined on enzyme hydrolysates of the protein preparations which were incubated for 72 hours. The amounts are given in per cent of protein corrected to the per cent nitrogen in the original sample; which was, for casein, 13.1 and, for lactalbumin, 13.8.

[3] Values in parentheses represent a decrease in per cent from that found in the original raw material.

[4] Determined by the ninhydrin method.

[5] Acetate content, 6%.

[6] The acetylated protein was reacted with lactose under the mild conditions of treatment.

[7] Acetate content, 3%.

available lysine, methionine, and tryptophan. There was not, however, a corresponding decrease in the rat growth value of lactalbumins combined with lactose under mild conditions. The ability of the animals to break the bonds releasing the am-

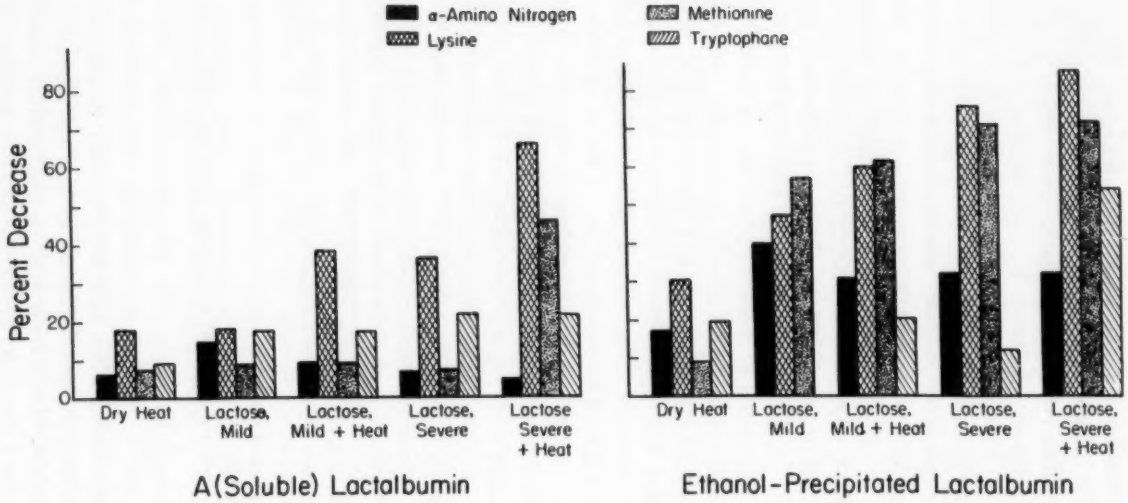

Fig. 1 The percentage decrease (based on the values for the raw protein) of α-amino nitrogen (ninhydrin method), lysine, methionine, and tryptophan in lactalbumins treated as follows: dry heat; combined with lactose under the mild and severe conditions of reaction; and each of these preparations heated. All values were measured in enzyme hydrolysates after 72 hours' incubation at 37°C. All heating was carried out at 150°C. for 70 minutes.

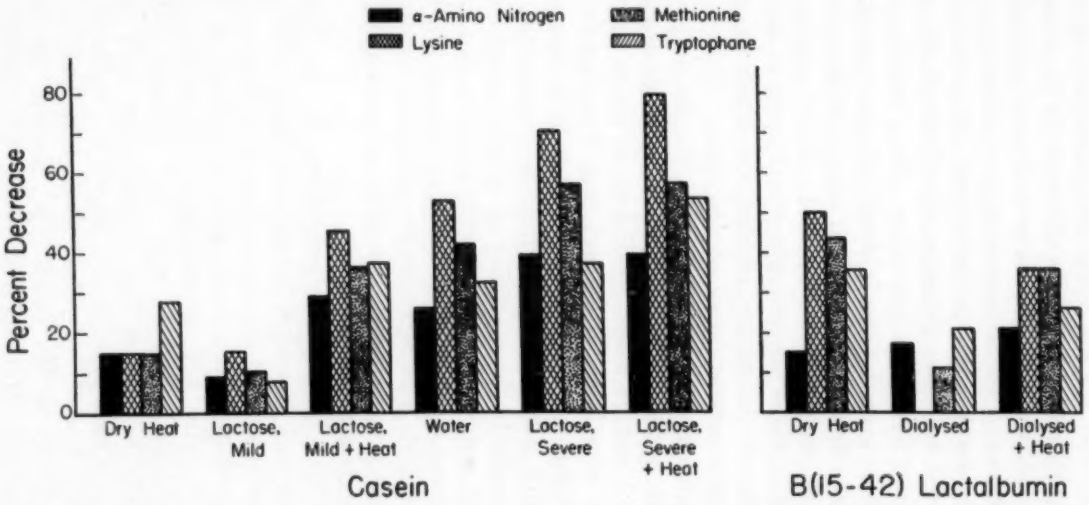

Fig. 2 The percentage decrease (based on the values of the raw protein) of α-amino nitrogen (ninhydrin method), lysine, methionine and tryptophan in proteins treated as follows: (a) casein — by dry heat, combined with lactose under the mild and severe conditions of reaction, and each of these preparations heated and refluxed in water at 95°C. for 24 hours; (b) B(15–42) lactalbumin heated, dialyzed, and dialyzed heated. All values were measured in enzyme hydrolysates after 72 hours of incubation at 37°C. All heating was carried on at 150°C. for 70 minutes.

ino acids in one and not the other lactose-combined protein would offer additional evidence of a difference in the extent of the initial reaction between these two proteins and lactose. Further heat treatment of the lactose-combined lactalbumins did, however, produce a decrease in the growth value for rats in proportion to the decreases found in microbiologically available amino acids. Friedman and Kline ('50a) found a much greater loss of rat growth value in dried, glucose-treated protein hydrolysates than microbiological assays of the constituent amino acids would have indicated. Their results and those obtained in these experiments would seem to indicate some variations in the ability of rats and assay microorganisms to utilize certain forms of proteins.

That the amino groups of the protein are the major site of reaction with lactose was shown by the acetylation experiments (tables 1 and 2) under conditions in which only the amino groups were blocked (Olcott and Fraenkel-Conrat, '47). The acetylation of casein succeeded only in decreasing its reaction with lactose. This finding, in conjunction with the difference noted in the reactivity of casein toward lactose under mild and severe conditions, would indicate that casein probably has some masked amino or other groups which become more available with increasingly strenuous conditions. Lactalbumin has given no such indication of possessing any masked amino groups.

When the above findings are considered in the light of those of Mohammed, Fraenkel-Conrat and Olcott ('49) with bovine serum albumin and glucose, of Lea and Hannan ('50) with sodium caseinate and glucose, and, by analogy, of those with various proteins and formaldehyde (Fraenkel-Conrat and Olcott, '48a, b; Fraenkel-Conrat and Mecham, '49), it seems reasonable to assume that several steps may be involved when these milk proteins are heated or combined with lactose and then heated: (a) Heat alone may lead to new cross-links, probably of the amide type, between the amino and carboxyl groups (Mecham and Olcott, '47; Mitchell and Block, '46); (b) under the mild conditions, the lactose reacts

with the free amino groups of the protein and gives rise to products which are only slightly resistant to enzymatic digestion; (c) one may reason by analogy that upon heating, after the initial protein-lactose reaction, or refluxing the protein and sugar, cross-links may be formed involving essentially the formation of a methylene bridge between an amino group and an amide or guanidyl group in the presence of an aldehyde, as has been shown to be the case when formaldehyde is the reactive agent; (c) at even higher temperatures, aldehydes can form cross-links between amino groups and the reactive $\geq$CH of phenol, imidazole, and indole groups; such links are resistant to acid hydrolysis (Fraenkel-Conrat and Olcott, '48b). Perhaps some such reaction may also take place in the more severe conditions of reaction here reported, where not only was enzymatic digestibility greatly reduced, but also acid hydrolysis failed to release the total amounts of the amino acids originally contained in the protein.

The products which have been described by Lea and Hannan ('50) in their three-step reaction between sodium caseinate and glucose appear to have properties similar to those of the products obtained in the present study, and the progressive decrease in available amino acids reported by them for each of these stages undoubtedly resulted from condensations and cross-links such as those described above. Friedman and Kline ('50b), in studying the reaction of essential amino acids with glucose, were able to isolate a highly fluorescent, colorless fraction which was not identical with the brown substance and had reduced biological value. They suggested that the formation of colored compounds is preceded by the production of fluorescent reaction products. Gottschalk and Partridge ('50) have also isolated a colorless amino acid-glucose product and identified it as the N-glucoside which can condense further with the formation of brown products. It seems plausible that these colorless products are analagous to those produced in the present study under the mild conditions of lactose reaction.

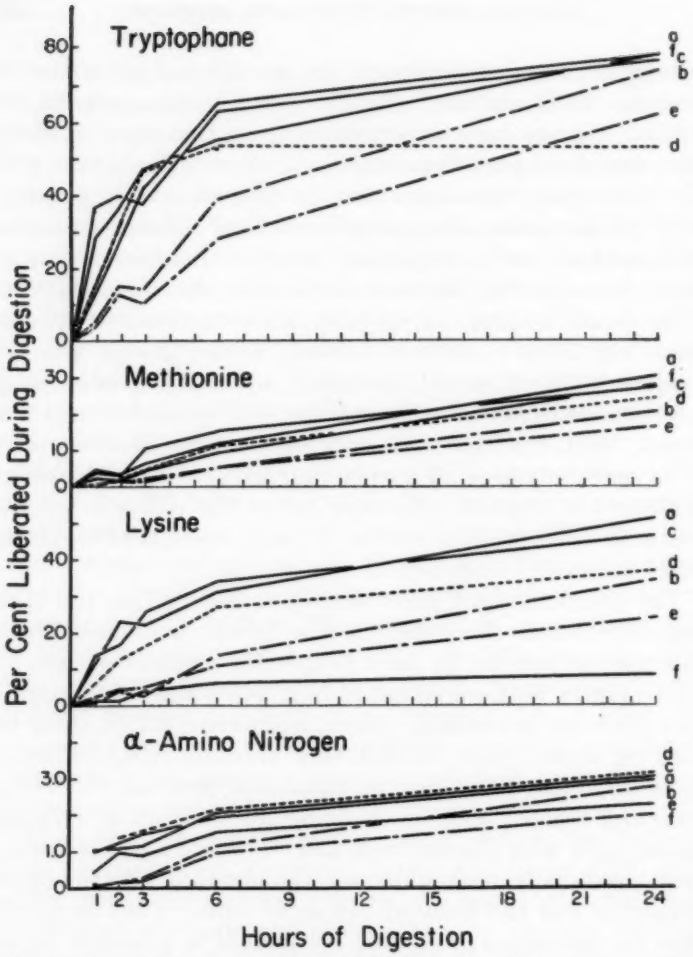

Fig. 3 The effect of heat or lactose combination or both on the enzymic (Pangestin) rate of release of α-amino nitrogen and amino acids in casein. The lactose combination was carried out under the mild conditions with a ratio of protein to lactose of 1: 0.3. The α-amino nitrogen was determined by the Van Slyke method.

(a) Unheated casein
(b) Casein heated at 150°C. for 70 minutes
(c) Casein combined with lactose, unheated
(d) Casein combined with lactose, heated at 118°C. for 30 minutes
(e) Casein combined with lactose, heated at 150°C. for 30 minutes
(f) Acetylated casein, acetate content 6%

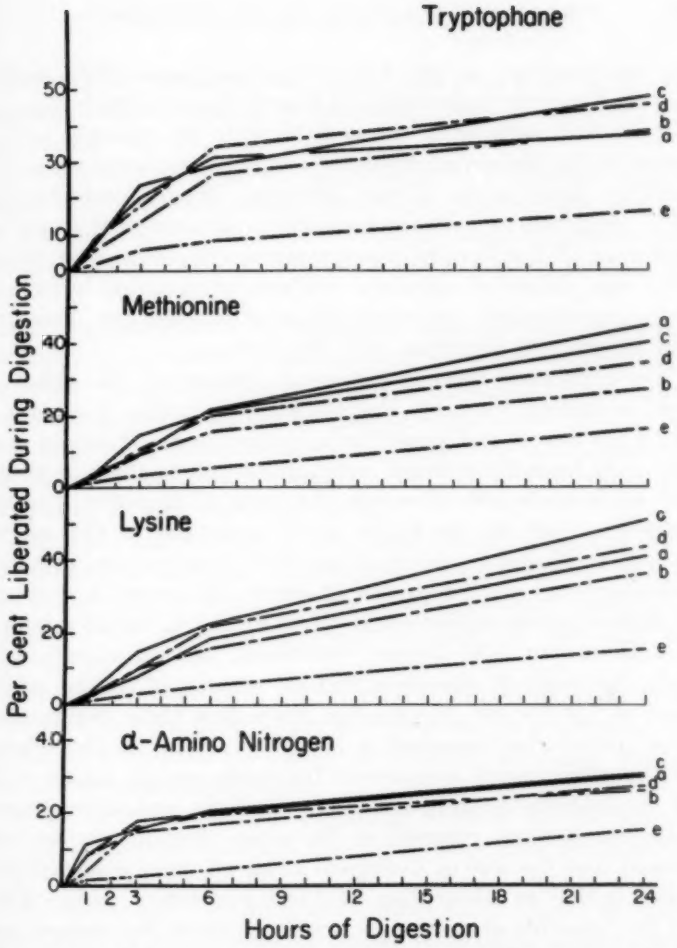

Fig. 4 The effect of heat or lactose combination or both on the enzymic (Pangestin) rate of release of α-amino nitrogen and amino acids in A(soluble) lactalbumin. The lactose combination was carried out under the mild conditions with a ratio of protein to lactose of 1: 0.3. The α-amino nitrogen was determined by the Van Slyke method.

(a) Unheated lactalbumin
(b) Lactalbumin heated at 150°C. for 70 minutes
(c) Lactalbumin combined with lactose, unheated
(d) Lactalbumin combined with lactose, heated at 118°C. for 30 minutes
(e) Lactalbumin combined with lactose, heated at 150°C. for 70 minutes

Since analyses of the various preparations after rather thorough enzyme hydrolysis (72 hours) failed to show changes that seemed sufficiently great to explain the amount of decrease in the biological efficiency of the proteins shown in the feeding experiments (Cook, Morgan, Singer and Parker, '51), experiments were carried out to determine the rate of digestion of the various preparations. The course of digestion was followed through release of α-amino nitrogen, lysine, methionine, and tryptophan after one, two, three, 6, and 24 hours of digestion with Pangestin.

The results of these experiments measuring the effect of heat or lactose, or both, are shown graphically in figures 3 and 4 for casein and lactalbumin, respectively. It can be seen that mild lactose treatment, when measured by these methods, did not significantly decrease the rate of digestion nor the release of any of the amino acids measured at the end of 24 hours. However, the first group of experiments, in which digestion was carried on for 72 hours, did reveal a decrease in total available amino acids. Heat at 118°C. for 30 minutes, in addition to mild lactose treatment, altered significantly both the rate of digestion and of release of amino acids; heat at 150°C. for 70 minutes intensified these differences. Heat alone also exercised a delaying action on the release of the amino acids measured. Inasmuch as the amino acids of the various protein-lactose derivatives and their heated products are not released at the same rate, it may be suggested that the loss of biological value of these modified proteins is due to absorption of their respective amino acids by the intestine at rates which will not allow for mutual supplementation. This would be in agreement with the *in vivo* experiments of Melnick and Oser ('49), in which the rate of release and absorption of lysine from heated casein was too slow to permit effective supplementation of other essential amino acids.

SUMMARY

The effects of heat or lactose or both on casein and several types of lactalbumins have been investigated.

These milk proteins have been shown to bind lactose in amounts ranging from 2 to 8% under different experimental conditions.

Casein, refluxed at 95°C. for 24 hours in a solution containing a ratio of protein to lactose of 1:1, bound twice as much lactose as when heated at 52°C. for the same length of time in a solution in which the ratio of protein to lactose was 1:0.3 or 3.5:5.

The two lactose-free lactalbumins used, one prepared commercially by an unknown process and the other a laboratory-prepared, ethanol-precipitated sample, combined with no more lactose when refluxed at 95°C. than when heated at 52°C. if the ratio of protein to lactose was more than 1:1. If the ratio of protein to lactose were decreased to 3.5:5 or 1:2, more lactose was bound by the protein at 52°C.

That free amino groups are essential for reaction with lactose was established by acetylation experiments. Acetylated lactalbumin reacted with no lactose present. Acetylated casein reacted with considerably less lactose than did the original protein.

Mild lactose treatment (52°C. for 24 hours) of these milk proteins rendered them somewhat resistant to enzymatic digestion as measured by release of α-amino nitrogen, lysine, methionine, and tryptophan.

Severe lactose treatment (refluxing at 95°C. for 24 hours) effected a further decrease in digestibility and availability of the same amino acids.

Heat alone had no appreciable effect on the total *in vitro* digestibility or availability of amino acids of A(soluble) lactalbumin but led to a decreased digestibility of casein and ethanol-precipitated lactalbumin.

Heat subsequent to reaction of the proteins with lactose greatly intensified the decrease in nutritive value of both casein and lactalbumin.

Heating of a commercial lactose-containing lactalbumin, B(15–42), had the same deleterious effects as heating the pure proteins after they had been reacted with lactose.

The various amino acids were released at different rates following heat or lactose treatment or both. It was suggested that this difference in the rate of release of certain essential amino acids may be sufficient to prevent their mutual supplementation in *in vivo* experiments.

Arguments were presented in support of the view that browning of milk proteins in the presence of lactose takes place in several steps and may lead to the formation of new cross-links, thereby accounting for the resistance of the protein derivatives to enzymatic attack.

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NITROGEN BALANCE INDEX IN THE ADULT RAT AS AFFECTED BY DIETS LOW IN L- AND DL-METHIONINE[1]

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TWO FIGURES

(Received for publication December 12, 1950)

Jackson and Block ('32) were the first to prove that both D- and L-methionine "stimulate growth" in the rat, and it is now generally accepted that the two isomers of this amino acid are metabolically equivalent. It was clearly demonstrated by Grau and Almquist ('43) that 0.25% supplements of DL- and L-methionine are equally effective in promoting growth in the chick. The work of Wretlind ('50), however, indicates that the D and L isomers are not utilized to the same extent for growth in young rats; D-methionine was found to be 76% to 84% as effective as the natural isomer. The results of Brush, Willman and Swanson ('47) demonstrate that DL-methionine added to a basal nitrogen-low diet will spare body nitrogen in the adult rat. Quantitative measurements of the utilization of the two isomers by the adult animal are not available. The purpose of the present investigation was to supply some of the missing information by comparing the utilization of DL- with L-methionine in the adult rat, as measured by a nitrogen balance method.

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METHODS

One group of adult male rats (series 180) served for all of the experiments except one dealing with DL-methionine. The data for the middle point on the DL-methionine curve in figure 2 were obtained with another group of animals (series 40). Ages at the beginning were 192 or 195 days. After a 48-hour fast body weights ranged from 228 to 286 gm (mean, 256 gm). All animals received a total of 43.9 Cal./rat/day (Calories = 121 $kg^{\frac{3}{4}}$). Each experiment included the following sequence of feedings: maintenance diet (9.6% whole egg protein), two weeks; nitrogen-free (N-free) diet, one week; amino acid diet, containing approximately half the maintenance requirement of nitrogen (half-N), one week; amino acid diet, containing approximately the maintenance requirement of nitrogen (full-N), one week. This 5-week cycle of feeding was repeated for each amino acid mixture investigated.

The complete amino acid mixture simulates the proteins of whole egg in that it contains approximately the same amount of each natural isomer of the essential amino acids per gram of total nitrogen. The non-essential amino acids of egg protein are replaced, in this mixture, by D-forms of 6 essential amino acids plus sufficient L-glutamic acid to make up to the same amount of total nitrogen.[3] The diet provided 11.5 mg choline chloride/rat/day but contained no cystine or cysteine. The detailed composition of the various diets and the methods used in this work are described in previous papers (Anderson and Nasset, '48, '50; Nasset and Anderson, '50).

The one-half DL-methionine amino acid mixture contained 22.7 mg of methionine nitrogen per gram of total nitrogen. The methionine omitted was replaced by an isonitrogenous amount of L-glutamic acid. The slight change in computed physiological heat value occasioned by this substitution was

[3] Amino acids and vitamins were donated by Merck and Company. Folic acid was donated by the Lederle Laboratories.

corrected by adjusting the sucrose content. The one-half L-methionine amino acid mixture also contained 22.7 mg of methionine nitrogen per gram of total nitrogen. In each instance, therefore, the fraction refers to the proportion of an amino acid in the mixture, regardless of the isomer present. Hence the one-half L-methionine mixture contained twice as much of the L-isomer as the one-half DL-mixture.

The N-free, half-N and full-N diets were fed by stomach tube in two equal portions daily. Nitrogen intake was determined by analyses of the dietary components as well as of the whole diet. The maintenance diet was weighed into feeding cups daily in amounts sufficient to provide 43.9 Cal./rat/day. It was always completely consumed. Feces were separated by Fe_2O_3 and Cr_2O_3 markers and preserved in sulfuric acid. Urine was collected on the last 4 days of each period and total N determined by the micro-Kjeldahl method. On the morning of the first day of urine collection the fine screen and collecting funnel is sprayed with approximately 10 ml of a 50% alcohol solution of benzoic acid (30 gm/l) and phenyl mercuric nitrate (0.5 gm/l). On the morning of the third day this process is repeated. In each instance the excess drains into the collecting flask below. It serves as an excellent preservative and prevents loss of nitrogen. If urine of known nitrogen content is sprinkled in the cages, without the presence of the animal, the recovery of nitrogen is 98% to 100%.

RESULTS

The body weights given in table 1 are averages of the weights at the end of the N-free, half-N and full-N periods in each experiment. The maximum change between adjacent periods was a gain of 3% in experiment V, in which the fat content of the diet was decreased from the usual 36% to 10% of calories from fat. The calorie deficit caused by this change was corrected by adding sucrose.

The fecal nitrogen within each experiment remained essentially constant, and according to convention this is taken to mean complete digestion and absorption of the nitrogenous

portion of the diet. In confirmation of previous work from this laboratory, the urinary nitrogen was appreciably diminished by the addition of a complete amino acid mixture to the N-free diet. This is true whether the full or the half maintenance quantity of nitrogen is added.

Nitrogen balance is plotted against nitrogen intake in figure 1. Each point represents the mean value for the group

TABLE 1

Average nitrogen balance data for rats receiving amino acid mixture diets (Series 180 rats)

EXPERIMENT NUMBER	I	II	III	IV	V
Amino acid mixture	Complete	1/2 DL-Methionine	1/2 L-Methionine	Complete	Complete (low-fat)
Number of rats	12	13	9	8	8
Body weight (kg)	0.259	0.258	0.265	0.263	0.271
Metabolic body size ($kg^{3/4}$)	0.363	0.362	0.370	0.367	0.376
	N balance data (mg N/day/$kg^{3/4}$)				
"N-free" period					
N intake	4	3	5	4	4
Fecal N	37	36	32	31	31
Urinary N	161	169	125	158	101
"Half-N" period					
N intake	75	74	74	71	69
Fecal N	38	38	31	30	31
Urinary N	116	156	128	107	89
"Full-N" period					
N intake	148	146	142	138	138
Fecal N	40	34	35	31	31
Urinary N	119	143	138	101	91

of animals as that given for the corresponding experiment in table 1. The topmost and middle points for each experiment represent full-N and half-N intake, respectively. The slope of the line joining these two points is equal to the fraction utilized of the increment in nitrogen intake. This coefficient of nitrogen utilization was given the symbol K′ by Allison et al. ('46). The lowest points in figure 1 represent the N-free periods. These points and the corresponding values for K′ are included to emphasize the fact that the nitrogen

balance data obtained with N-free diets in these experiments are too erratic to serve as a sound basis for computing the nitrogen balance index for the adult rat. For example, in experiment II (one-half DL-methionine) using the N-free and full-N periods, $K' = 1.19$; this is superior to the complete animo acid mixture used in experiment V, where the analagous $K' = 1.08$. For this and other reasons, detailed elsewhere (Nasset and Anderson, '50), the slope of the line joining

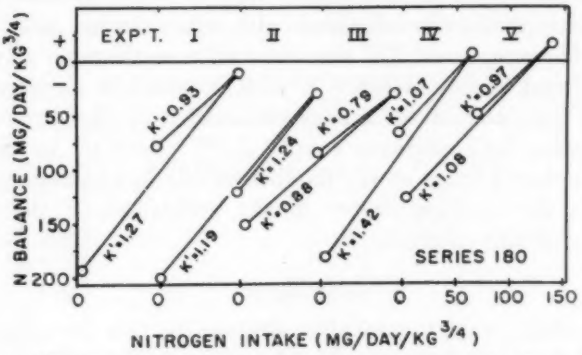

Fig. 1 Nitrogen balance index of ingested nitrogen, K', for amino acid mixtures:

Experiment I — Complete amino acid mixture
Experiment II — One-half DL-methionine
Experiment III — One-half L-methionine
Experiment IV — Complete amino acid mixture
Experiment V — Complete amino acid mixture with low fat.

the two uppermost points appears to be a better index of the quality of an amino acid mixture.

It seems worthwhile to emphasize the constancy of K' on the complete amino acid mixture when computed from the half-N and full-N data. The weighted mean K' in experiments I, IV and V is 0.98. Furthermore, this value does not seem to change with time on experiment; the interval between experiments I and V was 25 weeks. As might be expected on a low nitrogen intake over this extended period, the nitrogen balance, on the same intake, improved slightly and at a

uniform rate. This can be seen by laying a straight edge through either the full-N or the half-N points in experiments I, IV and V.

The values obtained for K′ by the method described above in experiments I, IV and V are not significantly different. This result was expected because the diets used in these experiments contained the complete amino acid mixture. Experiment II involved the use of the one-half DL-methionine mixture and yielded $K' = 1.24$; this is much greater than any value previously obtained with other amino acid mixtures. In experiment III the one-half L-methionine mixture was fed and in this instance $K' = 0.79$, which is significantly smaller than any of the values obtained with the three complete amino acid mixtures (expts. I, IV and V). As judged by the reduced value of K′, the low methionine in experiment III was the limiting factor in the utilization of the total nitrogen of this mixture.

DISCUSSION

It is clear from the results obtained in this investigation that K′ does not fully express the value to the animal of an amino acid mixture. The literature yields no evidence that a racemic mixture of an amino acid is nutritionally superior in any respect to an equal amount of the natural isomer, yet in experiment II (one-half DL-methionine) $K' = 1.24$, and in experiment III (one-half L-methionine) $K' = 0.79$. By definition K′ is the fraction of the increment in ingested nitrogen which is utilized to maintain nitrogen equilibrium. K′, therefore, is concerned only with the change in nitrogen balance which occurs when an experimental subject passes from a plane of low nitrogen intake to a higher one. In this sense the DL-methionine mixture was significantly superior to the mixture containing L-methionine ($P < 0.001$). The absolute nitrogen balance, however, cannot be ignored in considering the value to an animal of any source of nitrogen; in this respect the DL-methionine at half-N intake (expt. II, middle point in figure 1, N balance $= -120$ mg N/day/kg^{3}) is de-

cidedly inferior to the L-methionine at half-N intake (expt. III, middle point on graph, N balance = −85 mg N/day/kg$^{\frac{3}{4}}$). The nitrogen balance data for these two points were remarkably uniform and the difference is highly significant ($P < 0.001$). At full-N intake the nitrogen balances on the DL- and L-mixtures were identical (expts. II and III, topmost points on graph, N balance = −31 mg N/day/kg$^{\frac{3}{4}}$). When the absolute value of the nitrogen balance only is considered, it is evident that the DL- and L-methionine mixtures are equally effective at or near nitrogen equilibrium; when the nitrogen

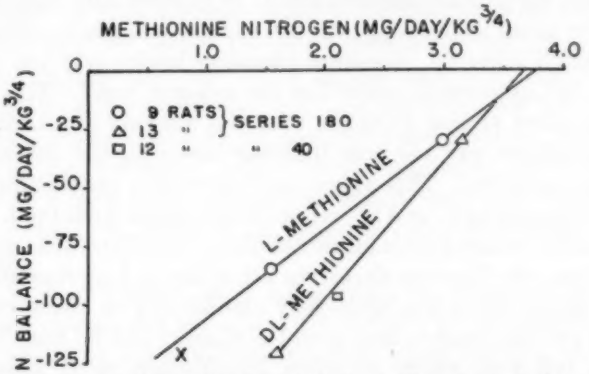

Fig. 2 Nitrogen balance plotted against the intake of methionine nitrogen (X = value computed assumng that only the L- component of the DL-mixture was utilized).

intake is reduced to approximately half of the maintenance requirement, the natural isomer is significantly superior to the racemic mixture.

Some of the relationships between the DL- and L-methionine mixtures are more clearly seen in figure 2, in which nitrogen balance is plotted against methionine nitrogen intake. Since K′ for the one-half L-methionine mixture is significantly less than unity (fig. 1), it is assumed that the methionine concentration in both experiments II and III was the limiting nutrient in both. This deduction is reasonable because there is no evidence that a racemic mixture of any amino acid is

superior metabolically to an isonitrogenous amount of the natural isomer.

Convincing additional support for the conclusions based on the experiments with DL-methionine has been gained from previous work done in this laboratory. About three years ago a nitrogen balance experiment was performed using a one-third DL-methionine mixture (Anderson and Nasset, '48). The mean result obtained with 12 rats was plotted as the middle point on the DL-methionine curve (the square in figure 2) and fell very close to the line joining the two points obtained in the present investigation. It is especially significant that the point established in the earlier work was obtained on a full-N diet; i.e., the total nitrogen intake was the same as for the uppermost point for the present work. This constitutes very strong evidence that the amount of DL-methionine available was the sole limiting factor in the utilization of these mixtures. This agrees with previous work using low concentrations of L- and DL-valine (Nasset and Anderson, '50).

It seems reasonably well-established, therefore, that methionine was the limiting factor in all of these experiments. If other points were available they should fall on these curves within certain limits; this would obviously not be true if the points fell well above nitrogen equilibrium or if they approached the point of zero nitrogen intake. As a consequence of the relationships discussed above, it may be permissible to extrapolate the lines joining the experimentally determined points to the point of zero nitrogen equilibrium in order to arrive at a value, under these conditions, for the daily requirement of methionine nitrogen for the maintenance of nitrogen equilibrium. This estimate for L-methionine nitrogen is 3.82 mg/day/kg$^{\frac{3}{4}}$; the corresponding estimate for DL-methionine nitrogen is 3.70. These values are essentially identical, and within the limitations mentioned above they reaffirm the current notion that the two isomers of methionine are metabolically equivalent. The average requirement for nitrogen balance is, therefore, 3.76 mg of methionine nitrogen or 40.0 mg of the amino acid/day/kg$^{\frac{3}{4}}$. The daily minimum

requirement for nitrogen balance reported by Rose ('49) for "normal man" is 1.10 gm. If it is assumed that the average normal man weighs 70 kg, the requirement of methionine as the amino acid is computed to be 45.5 mg/day/kg$^{.75}$. For this essential amino acid the requirements of the rat and man per unit of metabolic body size seem to be quite similar.

As is indicated in figure 2, the ingestion of approximately 1.5 mg of methionine nitrogen/day/kg$^{.75}$ as the natural isomer results in significantly greater nitrogen retention than the ingestion of the same amount of nitrogen as the racemic mixture. If it is assumed that D-methionine is not utilized at all at this low intake, the point plotted for the L-component of the racemic mixture (X in fig. 2) falls very close to the downward extrapolation of the line representing the experimental results obtained by feeding L-methionine. The data suggest the existence, under these conditions, of a sliding scale of D-methionine utilization which may vary from zero at low intake to 100% at or near the point of nitrogen equilibrium.

It is paradoxical that an animal in negative nitrogen balance should fail to utilize the D-form of methionine at a time when the need for this essential amino acid is so great. In this connection it is significant that Seifter et al. ('48) reported a greatly diminished D-amino acid oxidase in the livers of rats fed a non-protein diet. Miller ('48, '50) demonstrated in the rat that fasting or the feeding of either low protein or protein-free diets causes a drastic reduction in several liver enzymes. Rodney and Garner ('38) stated, without giving details, that the concentration of the D-isomer used as substrate exerted a "marked effect" upon the extent of deamination by rat liver or kidney. These studies suggest two possibilities for explaining the results of the present investigation: (1) the intake of methionine was so low that enzyme synthesis was diminished to a critical point; and (2) the concentration of D-methionine in the half-N experiment was so low that deamination and subsequent conversion

to the natural form was impossible. For the present no further speculation on the problem seems desirable.

In experiment IV, as in all previous experiments, fat contributed 36% of total calories; in experiment V fat contributed only 10% of total calories. The values for K′ in experiments IV and V were not significantly different (fig. 1), and hence the utilization of the complete amino acid mixture employed in both these exepriments was unaltered by the change in the fat content of the diet.

SUMMARY AND CONCLUSIONS

Nitrogen balance was determined on adult rats receiving amino acid mixtures as the sole source of nitrogen. Each experiment included: a 14-day period on a maintenance diet; a 7-day period on an N-free diet; a 7-day period on an amino acid mixture that supplied approximately half of the maintenance requirement of total nitrogen; and a 7-day period on the same amino acid mixture but with the quantity of nitrogen doubled. These diets, except the maintenance one, were fed by stomach tube in two equal portions daily and each rat received the same quantity of diet each day.

The weighted mean nitrogen balance index, K′, for three experiments (I, IV, V, in fig. 1) with the "complete" amino acid mixture was 0.98. When L-methionine was reduced to one-half of the amount in the complete amino acid mixture, $K' = 0.79$.

Data on nitrogen balance suggest that the D-component of DL-methionine is fully utilized at or near nitrogen equilibrium but may not be utilized at all when nitrogen balance is decidedly negative.

The requirement of methionine for maintenance of nitrogen equilibrium under these conditions is computed to be 3.76 mg of the methionine nitrogen/day/kg[1], or 40.0 mg as the amino acid. On the same basis this is very close to the requirement for man.

A change in the calories derived from fat from 36% to 10% failed to influence the utilization of the complete amino acid mixture.

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VITAMIN B_{12}, AN APF CONCENTRATE, AUREOMYCIN, STREPTOMYCIN, LIVER "L" AND FISH MEAL AND FISH SOLUBLES IN THE NUTRITION OF THE POULT[1]

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ONE FIGURE

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Stokstad and Jukes ('50b) reported that the feeding of aureomycin hydrochloride promoted the growth of White Holland turkey poults. Aureomycin, streptomycin, penicillin and sulfasuxidine have been shown to increase growth when added to the chick diet (Stokstad and Jukes, '50a; Whitehill et al., '50). The feeding of fermentation products of *Streptomyces aureofaciens* has been shown to increase chick growth (Stokstad et al., '49; Reed and Couch, '50), as well as to stimulate poult growth (McGinnis et al., '49; Singsen and Matterson, '49). There is little question but that crystalline B_{12} will increase the growth rate of chicks (Ott, '51; Nichol et al., '49). Attempts to stimulate the growth of turkey poults with crystalline B_{12} or an APF concentrate that contained only vitamin B_{12} have been unsuccessful (McGinnis et al., '49; Singsen and Matterson, '49; Stokstad and Jukes, '50b; Atkinson and Couch, '51; Patrick, '51).

Combs and Shaffner ('50) reported that the starting poult required an unidentified factor or factors present in dried brewers' yeast, menhaden fish meal and crab meal. Several

[1] Supported in part by grants-in-aid from the Lederle Laboratories, Pearl River, N. Y., and the Corn Products Refining Company, Argo, Illinois.

reports have been made to the effect that the growth obtained by feeding an all-vegetable protein ration to turkey poults could be improved by adding a source of animal protein (Hammond and Marsden, '39; Bird, Marsden and Kellogg, '48; Scott, Heuser and Norris, '48; Slinger et al., '49). Richardson and Blaylock ('50) obtained variable responses in poult growth by adding fish meal, fish solubles and an APF concentrate to an all-vegetable protein ration.

It should be mentioned that the results of the experiments reported in this paper are a continuation of work described in an earlier report (Atkinson and Couch, '51) and that such experiments were in progress when some of the work reviewed above was in press. The purpose of the experiments to be reported in the present paper was to determine the effect of feeding streptomycin, aureomycin, an APF concentrate (obtained from *Streptomyces aureofaciens* fermentation and designated as APF 4 in this paper), fish meal and fish solubles, and liver fraction "L" [2] on the growth, feed efficiency and mortality of growing turkey poults.

EXPERIMENTAL

The Broadbreasted Bronze poults used in this study were from the flock of the Poultry Department of the Texas Agricultural Experiment Station, College Station. The dams had been kept on litter and had been fed a diet containing, in per cent, 25 soybean oil meal, 5 fish meal, 5 dehydrated alfalfa leaf meal, 2½ bone meal, 2½ oyster shell, 0.5 salt, 15 whole oats, 25 ground yellow corn, 18½ ground milo, 0.5 fortified fish oil (3,000 A-400 D) and 0.25 APF concentrate 4 (described above). In addition, the ration was supplemented with 20 gm manganese sulfate, 45 gm "D activated" animal sterol [3] and 45 gm riboflavin concentrate [4] per 100 lb. of feed. The poults were hatched on March 23 and March 30, 1950. They were wingbanded, weighed and distributed at random among the

[2] Wilson and Company, Chicago, Ill.

[3] Delsterol, E. I. DuPont de Nemours and Co., New Brunswick, N. J.

[4] BY–21, Commercial Solvents Corporation, Terre Haute, Ind.

various groups of each experiment included in the present report and were maintained in pens (12 × 16 ft.) on pulp litter with electric brooders. The basal ration used in the present study contained, in per cent, 60 soybean oil meal, 34.75 ground yellow corn, 2.5 steamed bone meal, 2 oyster shell, 0.5 salt, and 0.25 fortified fish oil (3,000 A-400 D). In addition, 15 gm "D activated" animal sterol, 12 gm manganese sulfate and 50 gm riboflavin concentrate were added per 100 lb. of feed. This is the same diet used by Atkinson and Couch ('51) and designated diet L-1 by these workers. Other workers have also used soybean oil meal in the diet of turkey poults with satisfactory results (Funk and Kempster, '40; Hammond et al., '44; Bird, Marsden and Kellogg, '48; Combs and Shaffner, '50; Scott, '50). The level of protein (29.0 to 29.5%) used in diet L-1 is in agreement with the work of Scott, Heuser and Norris ('48) and Lloyd, Reed and Fritz ('49).

Six groups of 45 birds each were used in the first test (table 1). Groups 1 and 2 were fed the basal diet unsupplemented and, in addition, each poult in group 2 received a weekly injection of 2 μg of crystalline vitamin B_{12}.[5] This quantity of B_{12} (2 μg per bird per week) was thought to be adequate for turkey poults since Stokstad et al. ('49) had used 0.3 μg B_{12} per chick per week for the stimulation of chick growth. Group 3 received the basal diet supplemented with 66 mg of streptomycin and 13 μg of vitamin B_{12} per kilogram. Group 4 was fed 2% APF 4[6] substituted for an equivalent amount of ground yellow corn in the basal diet. Group 5 was fed 6% fish meal and 3% fish solubles, substituted for equivalent amounts of soybean oil meal in the basal diet. Group 6 received 4% liver "L"[7] in place of an equivalent amount of ground yellow corn in the basal diet.

[5] Merck and Co, Rahway, N. J.

[6] Supplied by the Lederle Laboratories, Pearl River, N. Y., and stated to contain 2 to 4 mg aureomycin per gram.

[7] Alcohol-insoluble fraction of liver which has been enzymatically digested. Supplied by the Wilson Laboratories, Chicago, Ill.

The second experiment consisted of 8 groups of 45 birds each (table 2). Groups 1 and 3 were fed the basal diet unsupplemented. In addition, each bird in group 3 was given a weekly injection of 2 μg of vitamin B_{12}. Group 2 was fed diet L-1 supplemented with 66 mg streptomycin and 13 μg B_{12} per kilogram.[8] Group 4 was fed diet L-1 plus 100 mg crystalline aureomycin hydrochloride per kilogram of diet.[9] Group

TABLE 1

The effect of feeding streptomycin, APF 4, fish meal and fish solubles, and liver "L" and injecting vitamin B_{12} on the weights of Broadbreasted Bronze poults kept on litter at 8 weeks of age

NO. GROUP	SUPPLEMENT TO BASAL RATION (L-1)	AVERAGE WEIGHT	FEED EFFICIENCY	MORTALITY
		gm	*gm*	%
1	None	1,139	2.6	2
2	Vitamin B_{12} injected (2 μg per bird per week)	1,177	2.5	2
3	MK-23 (66 mg streptomycin and 13 μg vitamin B_{12} per kg)	1,547	2.2	10
4	2% APF 4 (12 μg B_{12} per kg)	1,534	2.3	5
5	Fish meal (6%) and fish solubles (3%)[1]	1,255	2.7	16
6	Liver "L" (4%) (49.4 μg B_{12} per kg)	1,392	2.3	2

[1] Supplied 25.8 μg B_{12} per kilogram of diet.

5 was fed 100 mg of aureomycin per kilogram and in addition each bird received an injection of 2 μg of B_{12}. Group 6 was fed 6% fish meal and 3% fish solubles, substituted for equivalent amounts of soybean oil meal in the basal diet. Group 7 was fed 2% APF 4 substituted for an equivalent amount of ground yellow corn in the basal diet. Group 8 was fed 4% liver "L," substituted for an equivalent amount of

[8] Designated as MK–23 and supplied by Merck and Co.

[9] Supplied by the Lederle Laboratories.

ground yellow corn in the basal diet. All birds in the second experiment received the following added quantities of B vitamins, in milligrams per kilogram: thiamine 4, riboflavin 6, calcium pantothenate 15, niacin 100, pyridoxine 4, folic acid 2, biotin 0.2 and choline 2,000.

TABLE 2

The effect of feeding streptomycin, aureomycin, APF 4, liver "L", and fish meal and fish solubles, and injecting vitamin B_{12} on the weights of Broadbreasted Bronze turkey poults at 8 weeks of age

GROUP NO.	SUPPLEMENT TO BASAL RATION (L-1)	AVERAGE WEIGHT	FEED EFFICIENCY	MORTALITY
		gm	*gm*	%
1	None	1,391	2.6	8
2	MK-23 (66 mg streptomycin and 13 μg B_{12} per kg)	1,498	2.6	7
3	Vitamin B_{12} injected (2 μg per bird per week)	1,313	2.4	5
4	Aureomycin (100 mg per kg)	1,585	2.2	2
5	Aureomycin (100 mg per kg) and vitamin B_{12} injected (2 μg per bird per week)	1,740	2.0	6
6	Fish meal (6%) Fish solubles (3%)[1]	1,652	2.3	3
7	2% APF 4 (12 μg B_{12} per kg)	1,644	2.0	9
8	Liver "L" (4%) (49.6 μg B_{12} per kg)	1,539	2.3	5

[1] Supplied 25.8 μg B_{12} per kilogram of diet.

The vitamin B_{12} content of APF 4, fish meal, fish solubles and liver "L" was determined by the microbiological assay method of Skeggs et al. ('48) with *Lactobacillus leichmannii* 4797 (American Type Culture Collection) as the test organism. Thiomalic acid was used in the medium as a growth factor for *L. leichmannii*, as a reducing agent and for the protection of B_{12} during autoclaving. Acid production was the criterion used to measure the growth of the microorgan-

ism. The method described by Couch and Olcese ('50) was used to liberate the vitamin B_{12} in these products. Fish meal, fish solubles, liver "L" and APF 4 were found to contain 0.13, 0.6, 1.24 and 0.6 μg B_{12} per gram, respectively.

Diets were mixed biweekly. Feed and water were kept before the poults at all times. Birds were weighed weekly and observed daily for any symptoms or abnormalities. The duration of experiments 1 and 2 was 8 weeks; three groups of poults from experiment 2 were continued on test for a total of 14 weeks (table 3).

TABLE 3

The effect of feeding aureomycin and liver "L" and injecting vitamin B_{12} on the weights of Broadbreasted Bronze turkey poults at 14 weeks of age maintained on litter

GROUP NO.	SUPPLEMENT TO BASAL RATION (L-1)	AVERAGE WEIGHT	FEED EFFICIENCY	MORTALITY
		lb.	*lb.*	%
3	Vitamin B_{12} injected (2 μg per bird per week)	7.0	3.3	8
5	Aureomycin (100 mg per kg) and vitamin B_{12} injected (2 μg per bird per week)	8.8	2.8	6
8	Liver "L" (4%) (49.6 μg B_{12} per kg)	8.1	3.0	5

RESULTS AND DISCUSSION

First experiment

From the results obtained in the first experiment (table 1) it is quite apparent that the growth of the turkey poults was increased by the supplementation of the basal ration with streptomycin and B_{12} (group 3), with APF 4 (group 4), with fish meal and fish solubles (group 5) and with liver "L" (group 6). When the data of this test were treated statistically by analysis of variance an F value of 28.8 was obtained, whereas an F value of only 3.11 was necessary for significance

at the 0.01 level. Thus it is safe to state that the differences among the effects of the various diets are real.

The injection of crystalline vitamin B_{12} (2 μg per bird per week) failed to have any effect on the growth, feed efficiency or per cent mortality of the poults at 8 weeks of age. Supplementation of the basal diet with streptomycin and vitamin B_{12} (group 3) or APF 4 (group 4) produced a significant increase in the weights of the birds at 8 weeks of age and also improved the feed efficiency. The weights of the birds in the groups fed streptomycin and B_{12} (group 3) and APF 4 (group 4) were approximately the same and were significantly higher than the weights of the birds fed fish meal and fish solubles or liver fraction "L." The substitution of 6% fish meal and 3% fish solubles for an equivalent amount of soybean oil meal in diet L-1 (group 5, table 1) produced poults that weighed more than did those fed the basal ration unsupplemented, although this substitution in the basal diet did not improve the feed efficiency. The latter weight difference was significant at the 0.05 level but not at the 0.01 level. The substitution of 4% liver fraction "L" (group 6, table 1) likewise produced an increase in the weight of the birds when this group was compared with that fed the basal diet alone or the basal plus injected B_{12}. The growth obtained by feeding liver fraction "L" was significantly higher than that obtained when fish meal and fish solubles were fed (group 5). The feeding of liver fraction "L" improved the feed efficiency and was associated with a low mortality.

Second experiment

The results of the second experiment (table 2) show that supplementation of the basal diet (L-1) with streptomycin and B_{12} (group 2), aureomycin only (group 4), aureomycin and the injection of 2 μg crystalline B_{12} per bird per week (group 5), fish meal and fish solubles (group 6), APF 4 (group 7) or liver "L" (group 8) each produced a significant increase in the weights of the poults at 8 weeks of age. Treat-

ment of the data statistically by the analysis of variance gave an F value of 13.78, whereas an F value of only 2.73 was required for significance at the 0.01 level. Thus it is safe to conclude that the differences among the effects of the various diets are real.

Birds that weighed 1,740 gm at 8 weeks of age (table 2) were obtained by feeding aureomycin (100 mg per kilogram) and injecting vitamin B_{12} (2 μg per bird per week). These data apparently provided new information that was not forthcoming in the first test, since in this test one group had been included (group 4) where the antibiotic (aureomycin) was fed without vitamin B_{12}. Apparently the antibiotic precipitated a need for vitamin B_{12}, due to the increase in growth produced, which was not apparent in the absence of aureomycin. From the results of this test it may be noted that the growing poult does not require a source of vitamin B_{12} in the absence of an antibiotic. As a matter of fact, birds not injected with vitamin B_{12} weighed more than did those where the vitamin was injected. This observation is not considered to be of any consequence. These data seem to justify the following grouping of treatments, each composite grouping having produced a significant increase in the weight of the birds over the one just above it: First, groups 1 (basal only), 2 (injected B_{12} + streptomycin), and 3 (injected B_{12}); second, groups 6 (fish meal and fish solubles) and 7 (2% APF 4); third, groups 4 (aureomycin alone) and 8 (liver "L") and 4th, group 5 (aureomycin + B_{12} injected), which produced the heaviest birds in the test.

Experiments 1 and 2 described above were carried out concurrently and all groups in the second experiment were fed diet L-1 supplemented with 8 B vitamins. The results of the latter supplementation are shown in figure 1. From these data it is apparent that supplementation of the diet with added B vitamins increased the weights of the birds at 8 weeks of age in all cases with the exception of poults fed streptomycin and B_{12} (MK-23). Attention is also directed to the fact that the addition of B vitamins to a diet containing APF 4 did not

produce a significant increase in the weight of the poults. It seems quite possible to assume that the antibiotics (streptomycin in MK-23 and aureomycin in APF 4) were exerting a sparing effect on the B vitamin requirements, since supplementation of diets containing streptomycin or APF 4 failed to exert as great an effect on growth as was observed when other diets were supplemented with the 8 B vitamins. The

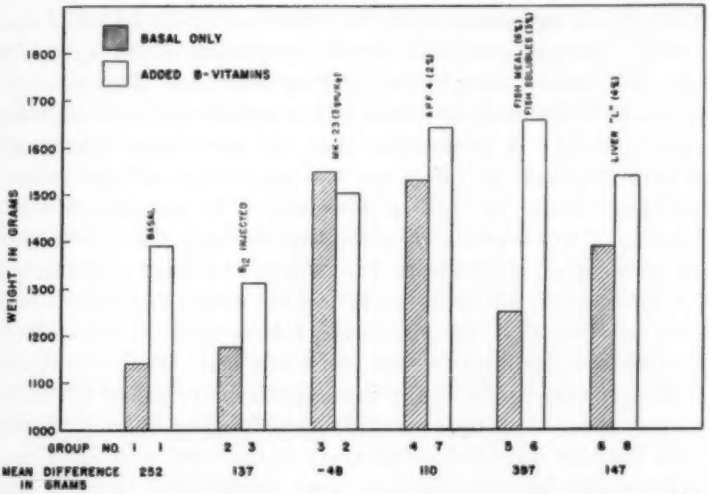

Fig. 1 Effect of adding 8 B vitamins to an all-vegetable protein diet (soybean oil meal-corn) in the presence of B_{12}, streptomycin and B_{12}, APF 4, fish meal and fish solubles, and liver "L."

greatest increases in weight which resulted from feeding the additional B vitamins were noted when the basal and the fish meal-fish solubles groups were each supplemented with the extra vitamins (fig. 1).

Groups 3, 5 and 8 of the second experiment were continued on test for an additional period of 6 weeks after the remaining groups were discontinued in an effort to determine whether the weight increases which were apparent at 8 weeks would continue to persist. These data are presented in table 3, and

there is little question but that the feeding of aureomycin (group 5) and liver "L" (group 8) continued to accelerate the growth of turkey poults up to and including the 14th week of the growth period.

From the data presented in experiments 1 and 2 (tables 1, 2 and 3) a few rather definite facts stand out. Crystalline aureomycin, as well as an APF concentrate which contained aureomycin, promoted the growth of turkey poults. This finding is in agreement with the report of Stokstad and Jukes ('50b). Streptomycin stimulated the growth of turkey poults but to a lesser extent than did aureomycin. The question arises as to the mode of action of the antibiotics in stimulating poult growth. It is possible that the antibiotics stimulated growth through an effect on the microflora of the gastrointestinal tract. If this be assumed, it is possible that the feeding of aureomycin or streptomycin may have decreased the number of unfavorable bacteria in the tract and allowed for an increase in the more favorable microorganisms. It is also possible that the favorable microorganisms may have synthesized unknown factors and made such factors available to the growing poult. A further hypothesis might be advanced to the effect that unfavorable bacteria may have competed with the host animal for nutrients in the tract and when such unfavorable microorganisms were eliminated, growth was accelerated. There is indication that streptomycin and the APF containing aureomycin (APF 4) stimulated the intestinal synthesis of B vitamins, since the addition of 8 B vitamins to a diet containing streptomycin (fig. 1) failed to stimulate the growth of the turkey poults, and stimulated that of poults fed a diet containing aureomycin APF to a much lesser extent than was noted in other comparisons (fig. 1).

Liver fraction "L" [10] promoted turkey poult growth in both experiments conducted (tables 1, 2 and 3). This product was included in the present study in an effort to find a crude

[10] See footnote 2, page 250.

material which would promote poult growth and which might serve as a source of unidentified factors required for the growth of the turkey. From the data obtained it is apparent that liver "L" did stimulate poult growth and may be considered to be a source of unidentified factors, since extra B vitamins were added with this material in the second experiment.

On the basis of the data of the second experiment it is believed that fish meal and fish solubles contain unidentified growth substances for the turkey. The latter may be deduced by assuming that the ingredients used in the basal diet supplied the amino acids necessary for the growing turkey. Since the growth obtained by supplementation of the basal diet with aureomycin and B_{12} (group 5, table 2) exceeded that obtained by supplementation with fish meal and fish solubles (group 6, table 2), it is therefore believed that the birds fed aureomycin and B_{12} (injected) were reasonably well-supplied with amino acids or protein since crystalline aureomycin was used. Fish solubles have been found to improve poult growth in one instance (German et al., '48) and failed to have any noticeable effect on the growth or mortality of young turkeys in another (Atkinson and Couch, '51).

Combs and Shaffner ('50) reported that the starting poult required an unidentified factor or factors present in dried brewers' yeast, menhaden fish meal and crab meal.

The injection of crystalline vitamin B_{12} (2 μg per bird per week) failed to have any effect on poult growth under the conditions of the experiments herein reported. Such an observation is in agreement with the earlier reports of McGinnis et al. ('49) and Stokstad and Jukes ('50b). It is fully realized that the injection of vitamin B_{12} at the rate of 2 μg per bird per week cannot be compared to the feeding of levels which varied from 12 to 49.4 μg B_{12} per kilogram of diet from crude sources (tables 1 and 2). It should be further emphasized that the above was taken into account in evaluating the data when liver "L" and fish meal and fish solubles were stated to contain unidentified factors for increasing the growth of

turkey poults. The level of B_{12} fed from crude sources was not considered to be of significance, since an earlier report from this laboratory (Atkinson and Couch, '51), as well as reports by other workers (McGinnis et al., '49; Singsen and Matterson, '49; Stokstad and Jukes, '50b; and Patrick, '51), have shown that the turkey poult does not respond to dietary additions of crystalline B_{12} or to an APF concentrate that contains vitamin B_{12} (without an antibiotic). It should be pointed out that the injection of 2 μg B_{12} per bird per week did produce a growth response in the presence of aureomycin.

The growth response obtained by supplementation with 8 B vitamins was particularly apparent in groups fed the basal diet unsupplemented and in those fed fish meal and fish solubles. Here it is fully realized that other workers (Jukes, '40a,b, '41; Evans et al., '42; Evans, '43; Patrick et al., '42; Singsen and Matterson, '50) have shown that choline promotes growth and prevents perosis. Perosis was not observed in the present study and it should be pointed out that Scott ('50) encountered perosis in turkey poults that received an all-vegetable protein ration. It should be realized that the poults used were probably of a different genetic constitution and that the poults used in the work of Scott ('50) may have been more susceptible to perosis than the ones used in the present study. Robblee and Clandinin ('50) reported that in order to prevent a deficiency syndrome characterized by poor growth, high mortality, broken feathers, dermatitis and perosis it was necessary to supplement practical turkey starting rations with calcium pantothenate and biotin. Such symptoms were not observed in the present study, although supplementation with 8 B vitamins did result in improvement in growth in the instances described above (fig. 1).

SUMMARY

From the results of this study it is apparent that the injection of crystalline B_{12} into poults fed an all-vegetable protein (soybean oil meal-corn) diet failed to have any effect

on growth, although such an injection did promote the growth of poults fed aureomycin.

Poult growth was stimulated by the feeding of aureomycin, streptomycin, an APF concentrate containing aureomycin, liver "L," and fish meal and fish solubles. It is believed that the increase in growth associated with the feeding of aureomycin and streptomycin was through an effect of the antibiotic on the microflora of the gastrointestinal tract. The APF concentrate, liver "L" and fish meal and fish solubles were postulated to contain unidentified factors required for poult growth.

Supplementation of the basal diet with 8 crystalline B vitamins promoted the growth of poults in some instances but failed to have an appreciable effect in the presence of streptomycin and B_{12} or when the aureomycin APF was fed. A possible explanation for the latter is presented. The data suggest that the favorable effect of the antibiotics on the microflora of the intestinal tract may have brought about additional synthesis of B vitamins, and that the latter may be a possible explanation for the failure of a growth response to B vitamins in the presence of streptomycin or aureomycin APF.

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THE INFLUENCE OF SEX AND DIET ON THE CAPACITY OF INTACT AND ADRENALECTOMIZED RATS TO ACETYLATE *p*-AMINOBENZOIC ACID[1]

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TWO FIGURES

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It has been reported previously that the survival of young adrenalectomized rats receiving adequate amounts of NaCl was significantly influenced by the calcium pantothenate content of the diet (Ralli, '46). On diets containing amounts of pantothenate adequate for intact rats (0.03 mg daily) the average survival was 14 days. However, when the intake of pantothenate was increased to 4 mg daily, the survival of the adrenalectomized rats was greatly prolonged, 50% surviving for more than 76 days. The growth of the rats in this group continued at an almost normal rate and their general condition was good (Dumm and Ralli, '48).

Pantothenic acid has been shown to be a constituent of coenzyme A (Lipmann et al., '47), which is essential in acetylation reactions, and to be present in cells largely as the coenzyme (Novelli et al., '49). Riggs and Hegsted ('48) observed that the ability of rats to acetylate injected *p*-aminobenzoic acid was decreased when the animals were fed a diet deficient in pantothenic acid. Shils, Seligman and Goldwater ('49) reported a similar decrease in the acetylation of sulfa-

[1] This research was aided by a grant from the National Vitamin Foundation.

nilamide. Other observers (Olson and Kaplan, '48) reported that the concentration of coenzyme A was low in tissues from pantothenate-deficient rats and ducklings.

In view of the importance of pantothenic acid in tissue reactions, and because of its influence on the survival of adrenalectomized rats, experiments have been carried out on the acetylation of *p*-aminobenzoic acid by intact and adrenalectomized rats. The influences of age, sex and the pantothenate content of the diet have been evaluated.

PROCEDURE AND RESULTS

Acetylation of *p*-aminobenzoic acid (PAB) by the rats was determined by the procedure of Riggs and Hegsted ('48). A dose of 1 mg of PAB in normal saline was injected intraperitoneally into the animal and urine was collected during the next 24 hours. Free and total PAB were estimated by the method of Bratton and Marshall ('39). The results are expressed as the per cent acetylated of the total PAB excreted in the urine. Unless at least 60% of the dose was recovered, the determination was excluded from the data to be presented.

Acetylation of PAB by intact rats

Young rats of both sexes were divided into three groups when they were 30 days of age. Group I was given the pantothenate-deficient diet (A); group II received the normal pantothenate diet (B) which supplied 0.1 mg of calcium pantothenate in 10 gm of diet; group III received the high pantothenate diet (C) which supplied 4 mg of pantothenate in 10 gm of diet. The composition of these diets is shown in table 1. The ability of the rats to acetylate was determined at weekly intervals.

The results of these experiments are shown in figure 1. Figure 1-B summarizes 63 determinations of the acetylation of PAB by 8 male and 7 female rats which were followed on the normal pantothenate diet from 31 to 119 days of age.

Figure 1-A shows the average of 94 determinations of the acetylation of PAB by 23 male and 21 female rats from 34 to 114 days of age. In figure 1-C is shown the average of 66 determinations of the acetylation of PAB by 10 male and 16 female rats which had been followed on the high pantothenate diet from 31 to 119 days of age. Each point represents an average of three to 14 determinations. The vertical lines represent the standard errors.

The ability of the rats on the normal pantothenate diet (fig. 1-B) to acetylate PAB increased in both sexes from 31

TABLE 1

Composition of experimental diets

VITAMIN SUPPLEMENTS TO 100 GM BASAL FOOD MIXTURE	DIET			INGREDIENTS OF BASAL FOOD MIXTURE	AMOUNT
	A	B	C		
	mg	*mg*	*mg*		%
Thiamine	0.3	0.3	0.3	Vitamin-free casein	22
Pyridoxine	0.3	0.3	0.3	Sucrose	62
Riboflavin	0.9	0.9	0.9	Hydrogenated cottonseed oil	9
Calcium				Cod liver oil	2
pantothenate	0.0	1.0	43.6	Mixed tocopherol	0.01
				Salt mixture [1]	5

[1] Composition of salt mixture: ferric citrate 2%, calcium phosphate (dibasic) 44%, potassium citrate 42%, magnesium citrate 12%, copper sulfate 0.09%, potassium iodide 0.005%.

All rats received 1% NaCl as drinking water.

to 65 days of age. After 65 days the acetylation of PAB remained at about 72% for males and about 64% for females on the normal pantothenate diet. The values reported for males agree closely with those reported by Riggs and Hegsted ('48) for normal male rats on a diet supplying slightly more calcium pantothenate (0.25 mg/10 gm diet).

The acetylation of PAB by pantothenate-deficient rats (fig. 1-A) was lower in all age groups than that of normal rats of the same age and sex (fig. 1-B). Of the rats which continued to survive on the pantothenate-deficient diet, a tendency to spontaneous recovery of the capacity to acetylate was observed in some animals.

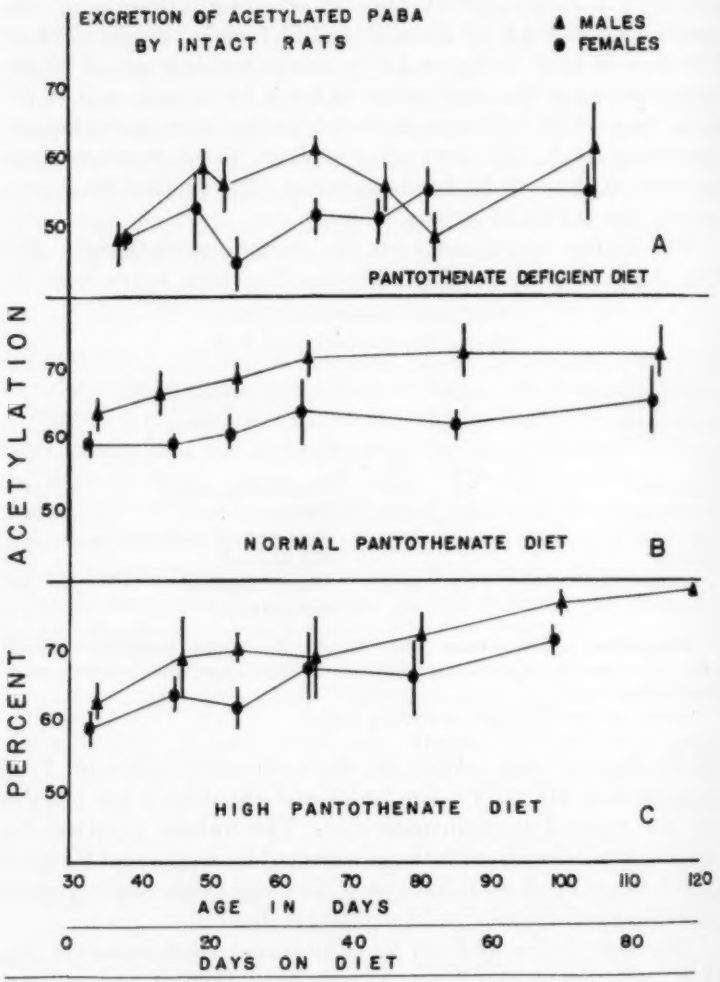

Fig. 1 The average per cent acetylation of PAB by intact rats is shown on (A) a pantothenic-deficient diet, (B) a normal diet (0.1 mg pantothenate in 10 gm diet), and (C) a high pantothenate diet (4 mg pantothenate in 10 gm diet). All experimental diets were begun at 30 days of age. The vertical lines represent the standard errors.

The average per cent acetylation by male rats on the high pantothenate diet (fig. 1-C) was approximately the same as that by male rats on the normal pantothenate diet until 80 days of age, after which the average acetylation of PAB by the high pantothenate rats was somewhat, but not significantly ($t = 1.238$; $p = 0.2$–0.3), greater than that of the rats on the normal pantothenate diet.

The average acetylation of PAB by female rats on the high pantothenate diet was consistently slightly greater than that of female rats of the same age on the normal pantothenate diet. The differences were not great, and for a single age group are of doubtful significance. When data from all female rats above 40 days of age were combined, the average acetylation of PAB by the high pantothenate rats was 65.5 $\pm$ 1.6%, compared with 61.7 $\pm$ 1.3% by female rats on the normal pantothenate diet ($t = 1.811$; $p = 0.05$–0.1).

In all diet groups the male rats excreted a larger proportion of the PAB in the acetylated form than did the female rats. Pantothenate-deficient male rats between 50 and 60 days of age acetylated 55.7 $\pm$ 3.9% of the PAB excreted, compared to 44.5 $\pm$ 3.9% by the female rats. In the same age group male rats on the normal pantothenate diet acetylated 68.7 $\pm$ 2.2%, compared with 60.7 $\pm$ 2.8% by the females. On the high pantothenate diet male rats between 50 and 60 days of age acetylated 70.1 $\pm$ 1.7% of the PAB excreted, compared with 61.9 $\pm$ 2.9% by the females.

Acetylation of PAB by adrenalectomized rats

Young male rats were placed on the pantothenate-deficient diet (A) when they were 30 days of age and were continued on this diet for 30 days. At this time half the rats were adrenalectomized and the remaining rats kept intact. After adrenalectomy half the adrenalectomized and half the intact rats were transferred to the high pantothenate diet. The remaining rats were continued on the pantothenate-deficient diet. The ability of the rats to acetylate PAB was determined at

intervals separated by at least three days. As a check on the completeness of adrenalectomy, salt was withheld from the diet of the adrenalectomized rats at the conclusion of the experiment. The adrenalectomized rats all died after salt was withheld.

The results are summarized in figure 2, in which a clear bar represents the average acetylation of the intact rats and a solid bar the acetylation of the adrenalectomized rats. In

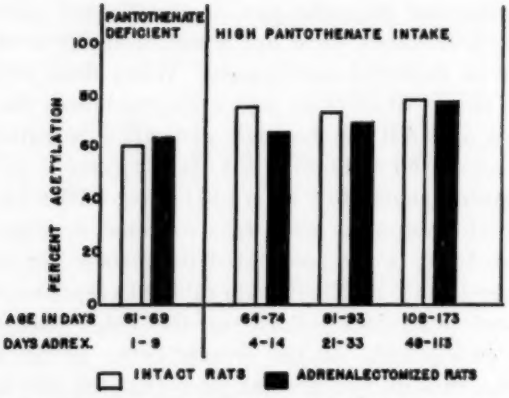

Fig. 2 The average per cent acetylation of PAB by intact (clear bar) and adrenalectomized (solid bar) rats continued on the pantothenate-deficient diet from one to 9 days after adrenalectomy is shown to the left. In the remainder of the figure is shown the average acetylation by intact and adrenalectomized rats on the high pantothenate diet from 4 to 14, from 21 to 33 and from 48 to 113 days after adrenalectomy. All rats received the pantothenate-deficient diet for 30 days before adrenalectomy.

the first section of the figure, the per cent acetylation of adrenalectomized and intact rats continued on the pantothenate-deficient diet is shown. Both groups excreted less PAB in the acetylated form than do normal male rats, with no significant difference ($t = 1.17$; $p = 0.2$–0.3) between the groups. The experiment could not be continued further with the adrenalectomized pantothenate-deficient rats due to the limited survival of these animals.

In the remainder of the figure is shown the per cent acetylation of PAB by adrenalectomized and intact rats transferred to the high pantothenate diet after adrenalectomy at 60 days of age. From 4 to 14 days after adrenalectomy, 6 intact male rats excreted an average of $74.8 \pm 1.2\%$ of the PAB in the acetylated form, compared with $66.2 \pm 3.0\%$ by 7 adrenalectomized rats ($t = 2.18$; $p = 0.05$–0.1), a difference which may be significant. From 21 to 33 days after adrenalectomy, the 5 intact rats acetylated $73.6 \pm 2.9\%$ of the PAB excreted, compared to $69.4 \pm 3.9\%$ by 8 adrenalectomized rats. After 48 to 113 days on the high pantothenate diet, the intact rats acetylated $77.9 \pm 2.8\%$ of the PAB, compared with

TABLE 2

Acetylation of PAB by adrenalectomized rats following salt withdrawal

NUMBER OF DETERMINATIONS	DAYS AFTER NaCl STOPPED	AVERAGE % ACETYLATION ± S. E.
5	..	72.8 ± 2.4
3	1	76.0 ± 1.7
3	2	80.7 ± 4.4
3	3	76.0 ± 6.7

$76.8 \pm 1.5\%$ acetylated by the adrenalectomized rats. Apparently the intact rats recovered the ability to acetylate PAB more rapidly after a period of pantothenate deficiency than did the adrenalectomized rats. However, after more than 20 days on a high pantothenate diet no significant difference was found between the intact and adrenalectomized rats.

In an effort to evaluate the effect of severe adrenal insufficiency, acetylation was determined in 6 adrenalectomized rats on the high pantothenate diet following salt withdrawal. The situation in the animal made it impossible to carry on any prolonged observations under these circumstances, as the rats did not survive salt withdrawal for more than three days. The results are shown in table 2, and it is clear that even during this period of acute adrenal insufficiency the capacity to acetylate PAB was maintained.

DISCUSSION

These observations have brought out the fact that intact male rats tend to acetylate PAB better than do female rats of the same age group. The difference between the sexes was greatest in rats from 50 to 70 days of age on the pantothenate-deficient diet. When the diet was excessively high in pantothenate, the capacity of the females to acetylate improved somewhat but a significant difference still occurred. One factor which possibly contributed to the difference in acetylation between the sexes may have been the greater weight of the male rats. However, this apparently was not the only explanation, as acetylation of PAB by young male rats exceeded that by the older and heavier females.

The adrenalectomized rats continued on the pantothenate-deficient diet survived only for a limited period. During this period their ability to acetylate was lower than normal but not lower than that of intact rats on the same diet. Pantothenic acid deficiency definitely limits the survival of the adrenalectomized rats, and it may be that the diminished capacity to acetylate associated with the deficiency contributes to the limited survival. This suggestion is supported by observations of the animals transferred to the high pantothenic acid diet after adrenalectomy. Improvement in the capacity to acetylate PAB occurred less rapidly in the adrenalectomized rats during the first two weeks after adrenalectomy, and after the diet was supplemented with pantothenate, than it did in the intact rats similarly treated. As the period of high pantothenate intake continued after adrenalectomy, the adrenalectomized rats regained the capacity to acetylate PAB to the same extent as did the intact rats.

It seems justifiable to state that the rat's ability to acetylate PAB does not depend on the presence of intact adrenal glands. This is further borne out by the acetylation studies done during the period of salt withdrawal in the adrenalectomized rats on the high pantothenate diet. Although these experiments could be done for only a few days, as the rats died of

adrenal insufficiency, the ability to acetylate was well maintained. These observations confirm the studies of Gershberg and Kuhl ('51) on human subjects with Addison's disease.

SUMMARY

Acetylation studies were done on intact male and female rats from 30 to 120 days of age on a normal diet, on a pantothenate-deficient diet and on a pantothenate intake of 4 mg daily. On all diets male rats acetylated a greater percentage of the injected PAB than did female rats. Acetylation was decreased in both sexes on the pantothenate-deficient diet.

Acetylation was also studied in adrenalectomized and intact male rats on a pantothenate-deficient or a high pantothenate diet following a 30-day period of pantothenate deficiency prior to adrenalectomy. Both intact and adrenalectomized rats, continued on the pantothenate-deficient diet, acetylated less PAB than do normal rats. When 4 mg of pantothenate were added to the diet, the capacity of the intact rats to acetylate PAB increasd rapidly, whereas the ability of the adrenalectomized rats to acetylate was restored more slowly. After 40 days on the high pantothenate intake the adrenalectomized rats had fully recovered their capacity to acetylate.

When the adrenalectomized rats on the high pantothenate diet were deprived of salt, so that severe adrenal insufficiency occurred, acetylation was still normal.

ACKNOWLEDGMENT

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THE ENERGY EXPENDITURE FOR QUIET PLAY AND CYCLING OF GIRLS 6 TO 14 YEARS OF AGE [1]

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This paper reports the results of measuring the energy expended by girls 6 to 14 years of age: (1) while sitting at quiet play such as cutting out pictures, coloring, pasting, and so forth; and (2) while cycling on a bicycle ergometer of electric brake type at an average rate of 56 pedal revolutions per minute against the resistance produced by a current of 0.6 ampere. These measurements were made in the respiration chamber of the Nutrition Laboratory of Teachers College, Columbia University, New York City. Reports found in the literature with which the present results could be compared are cited under "Results and Discussion."

EXPERIMENTAL

Twenty girls from two well-regulated homes for children served as subjects. All were born in America, the majority in

[1] The data on groups I and II are from dissertations submitted by Mary E. Robertson ('42) and Ethel M. Thompson ('40) in partial fulfillment of the requirements for the degree of Doctor of Philosophy under the Joint Committee on Graduate Instruction, Columbia University. The determinations on group III were made by Edna M. McIntosh while a graduate student in nutrition at Teachers College, Columbia University.

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New York City, but they were varied in racial background. All were in good health, attending school regularly. Deviations of body weight from the Baldwin-Wood predictions are shown in table 1 and range from — 14.8 to + 23.5%.

The basal metabolism was determined by means of the Sanborn-Benedict portable respiration apparatus, the Collins-Benedict-Roth respiration apparatus, or the Benedict and Benedict student respiration apparatus, following the procedure described in the paper from this laboratory by Taylor et al. ('48). The average results expressed in 4 ways are shown in table 1. The deviations of the basal metabolism results were, with very few exceptions, within the commonly accepted normal range of ± 15% when compared with the values predicted by the various accepted standards for children (Harris-Benedict, Dreyer, Benedict, Lewis et al., Mayo, and Talbot).

The activity studies, as has been said, were carried out in a respiration chamber. A full description of the chamber, the gas analysis procedure and method for sampling the chamber air, and the bicycle ergometer will be found in the paper by Taylor et al. ('48).

PROCEDURE

The procedure followed in these determinations was identical with that described in the paper just cited.

RESULTS AND DISCUSSION

The average results of 54 determinations of the energy expenditure for quiet play and of 55 for cycling, including the cost of the basal energy metabolism, are given in table 2 along with the standard errors of the mean and the coefficients of variation. Coefficients of variation ranging from 6.0 to 18.2% are easily accounted for by the variability of involuntary movements of head, hands and feet observed from day to day. That the range of the coefficients of variation is lower in the cycling than in the quiet play period is doubtless due

TABLE 1
Average weight, height, deviation from predicted weight, and basal metabolism of girls (figures in parentheses show range)

TABLE 1

Average weight, height, deviation from predicted weight, and basal metabolism of girls (figures in parentheses show range)

SUBJECTS	BODY WEIGHT		BODY HEIGHT		DEVIATION FROM PREDICTED WEIGHT [1]	BASAL METABOLISM WITH STANDARD ERROR OF THE MEAN CALORIES PER			
						24 hr.	kg/hr.	m^2/hr.	cm of ht./ 24 hr.
	lb.	*kg*	*in.*	*cm*	%				
Group I: 8 subjects with ages ranging from 6–0 to 8–8 [2]	52.5 (41.8–76.1)	23.9 (19.0–34.6)	47.9 (44.8–51.1)	121.7 (113.8–129.8)	— 0.5 (— 14.6–+ 23.5)	1017 ± 43	1.81 ± 0.08	47.5 ± 1.5	8.4 ± 0.3
Group II: 6 subjects with ages ranging from 9–9 to 11–8 [2]	68.0 (52.8–84.7)	30.9 (24.0–38.5)	54.6 (51.3–59.3)	138.6 (130.2–150.6)	— 7.0 (— 14.8–+ 1.9)	1049 ± 34	1.41 ± 0.05	39.6 ± 0.8	7.5 ± 0.1
Group III: 6 subjects with ages ranging from 11–6 to 14–2 [2]	102.3 (94.4–111.1)	46.5 (42.9–50.5)	60.8 (57.7–62.0)	154.4 (146.5–157.6)	+ 1.9 (— 7.5–+ 10.5)	1339 ± 25	1.21 ± 0.04	39.4 ± 1.0	8.7 ± 0.1

[1] Deviations from Baldwin-Wood standards.

[2] The first number in each set refers to years, the second to months.

TABLE 2

Average energy expenditure of girls for quiet play and cycling (including the basal metabolism)

	QUIET PLAY			CYCLING		
	Group I (6–9 yrs.)	Group II (10–12 yrs.)	Group III (12–14 yrs.)	Group I (6–9 yrs.)	Group II (10–12 yrs.)	Group III (12–14 yrs.)
CO_2 (gm) produced per girl per hr.						
Mean ± S.E.[1]	23.21 ± 0.65	25.37 ± 0.41	29.22 ± 0.91	51.54 ± 1.18	52.61 ± 0.74	52.31 ± 1.19
No. of cases	20	20	14	20	21	14
C.V.[2] (%)	12.5	7.3	11.6	10.2	6.5	8.5
Cal. per kg per hr.						
Mean ± S.E.	2.80 ± 0.11	2.38 ± 0.04	1.85 ± 0.06	6.19 ± 0.16	4.93 ± 0.09	3.31 ± 0.07
No. of cases	20	20	14	20	21	14
C.V. (%)	18.2	8.4	11.9	11.8	8.5	8.2
Cal. per cm of ht. per hr.						
Mean ± S.E.	0.57 ± 0.02	0.55 ± 0.01	0.57 ± 0.02	1.27 ± 0.02	1.14 ± 0.02	1.01 ± 0.02
No. of cases	20	20	14	20	21	14
C.V. (%)	14.0	6.0	10.5	8.7	6.1	7.9

[1] Standard error.

[2] Coefficient of variation.

to the activity in the cycling period being somewhat more controlled.

It will be noted that the energy expenditure for both quiet play and cycling expressed as Calories per kilogram per hour decreases with increase in age. The same is true of the energy expended per centimeter of height in cycling, but for quiet play the expenditure is practically the same in the three age groups. The figures for boys expressed as Calories per kilogram per hour reported by Taylor et al. ('48) also showed decrease with increase in age for both activities, but when expressed as Calories per centimeter of height there was

TABLE 3

Comparison of energy expenditure of girls and boys for quiet play and cycling

	GIRLS 6–9 YRS.	BOYS 7–8 YRS.	GIRLS 10–12 YRS.	BOYS 9–11 YRS.	GIRLS 12–14 YRS.	BOYS 12–14 YRS.
Cal. per kg per hr.						
Quiet play	2.8	3.1	2.4	2.6	1.9	2.1
Cycling	6.2	6.6	4.9	5.1	3.3	4.5
Cal. per cm of ht. per hr.						
Quiet play	0.57	0.59	0.55	0.58	0.57	0.58
Cycling	1.27	1.23	1.14	1.15	1.01	1.24

practically no change with increase in age. For ease of comparison the results on the boys of Taylor et al. ('48) and the girls of this study are brought together in table 3.

When the total cost of quiet play per kilogram per hour is compared with the basal energy expenditure, the percentage increase for group I is 55%, for group II 69%, and for group III 53%. For cycling the increases are, respectively, 242, 250, and 174%. When comparisons are made on the basis of Calories per centimeter of height per hour, the percentage increases for quiet play are respectively 63, 77, and 58%, while for cycling they are respectively 263, 268, and 181%.

No reports on the energy expenditure of girls in cycling have been found in the literature. For girls sitting quietly a

few comparable reports have been found. Speck (1892) reported on a series of determinations of the energy expenditure of a 10-year-old girl sitting quietly reading at a period 5 hours after breakfast which gave an average value of 1.98 Cal. per kilogram per hour. Sondén and Tigerstedt (1895) reported results on groups of girls ranging from 7 to 14 years in age. They were described as sitting quietly reading and frequently eating small amounts of candy or apples. The average result for girls of the age range of group I of this study was 2.46 Cal. per kilogram per hour, for those in the age range of group II it was 2.07, and for those in the age range of group III, 1.73. Bedale ('23), for girls 10 to 12 years of age sitting quietly in the schoolroom, reported an average value of 2.27 Cal. per kilogram per hour and for girls 12 to 14 years of age, 1.97 Cal. per kilogram per hour. Potgieter ('33), working with six 9-year-old girls sitting at quiet play in the respiration chamber, obtained an average of 2.98 Cal. per kilogram per hour.

SUMMARY

Fifty-four determinations of the energy expenditure for quiet play and 55 for cycling by 20 girls, 6 to 14 years of age, are reported. A respiration chamber equipped with a bicycle ergometer of electric brake type was used.

Twenty determinations were made on girls 6 to 9 years of age. For quiet play the average total energy expenditure was 2.80 Cal. per kilogram per hour, or 0.57 Cal. per centimeter of height per hour. These results are, respectively, 55 and 63% above the average basal energy expenditure. For cycling the average total expenditure was 6.19 Cal. per kilogram per hour or 1.27 Cal. per centimeter of height per hour. The increases above the basal expenditure are, respectively, 242 and 263%.

Twenty determinations of the energy expenditure for quiet play and 21 for cycling on girls 10 to 12 years of age gave for quiet play an average expenditure of 2.38 Cal. per kilogram per hour, or 0.55 Cal. per centimeter of height per hour, and

for cycling 4.93 Cal. per kilogram per hour or 1.14 Cal. per centimeter of height per hour. These results are, respectively, 69, 77, 250 and 268% above the basal expenditure.

Fourteen determinations were made on girls 12 to 14 years of age. The average energy expenditure for quiet play was 1.85 Cal. per kilogram per hour or 0.57 Cal. per centimeter of height per hour. These results are, respectively, 53 and 58% above the basal energy expenditure. For cycling the average was 3.31 Cal. per kilogram per hour or 1.01 Cal. per centimeter of height per hour. The increases above the basal expenditure are, respectively, 174 and 181%.

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BIOLOGICAL VALUE OF AN ENZYMATIC DIGEST OF BOVINE PLASMA [1]

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The biological evaluation of protein preparations intended for parenteral use in human nutrition by tests in experimental animals leaves much to be desired. It is obvious at the outset that the capacity of a protein product to promote the formation of tissue in immature or depleted experimental animals does not provide a completely valid estimation of the product's value for the biosynthesis of human tissues (Frost, '50). A study of well-substantiated species differences in amino acid needs and physiology reveals the limited usefulness of animal tests for these purposes (Mitchell, '50; Cox et al., '47). In view of these considerations, an investigation of the oral and parenteral nutritive value of an enzymatic digest of bovine plasma [2] was undertaken in infants and adults, respectively. The results of this study are here reported.

EXPERIMENTAL

I. Oral assay in infants

It is common knowledge that on a body weight basis the protein requirements of the infant are 4 to 5 times greater than those of the healthy adult. Because of the N losses which

[1] The work described in this report was supported by funds from the Office of Naval Research and Baxter Laboratories, Inc.

[2] Travamin.

prevail during illness, especially in fever and traumatic episodes, the protein needs of the convalescent adult may approximate in magnitude those of the normal infant. Since both conditions call for the formation of new human tissues, it seems reasonable to assume that a protein or protein product which will support good infant growth will also promote good tissue regeneration in the depleted adult.

TABLE 1

Composition and caloric distribution of diets

Evaporated milk formula (EM diet):			
Evaporated milk	50 ml		70 cal.
Corn syrup	10 ml		30 cal.
Water	40 ml		
Total	100 ml		100 cal.
Bovine plasma digest (Travamin) formulae:[1]			
(5% digest used in 5 BP diet and 15% digest used in 15 BP diet)			
0.56 gm digest N × 6.25		3.5 gm	14.0 cal.
Laboratory Product no. 217[2]		18.0 gm	
Fats	4.0 gm		36.0 cal.
Carbohydrates	11.6 gm		46.5 cal.
Brewers' yeast		1.0 gm	3.5 cal.
Water		88.0 ml	
Total		100.00 ml	100 cal.

Salt supplement, mg per 100 ml: $FeSO_4$, 10; NaCl, 100; Ca gluconate, 790; KH_2PO_4, 323; $Ca(OH)_2$, 197; K_2HPO_4, 108; KCl, 90; MgO, 18.

[1] We are indebted to Dr. Daniel R. Borgen of the Baxter Laboratories for supplies of these special products.

[2] The Laboratory Product no. 217 and brewers' yeast were kindly provided by Dr. Warren M. Cox, Jr., of Mead Johnson and Co.

Diets and methods. The composition and caloric distribution of the evaporated milk, 5 and 15% bovine plasma digest formulae employed in this study are shown in table 1. The 15% product contained added isoleucine and methionine. In the 5% product 55.8% of the total N was present as free amino N and in the 15% product 57% of the total N occurred

as free amino N. The diets were given in 5 feedings daily at the rate of 100 cal. and 0.56 gm of nitrogen per kilogram of body weight and were supplemented daily with 50 mg of ascorbic acid and 15 drops of oleum percomorpheum.

Owing to uncertainties regarding the B complex vitamins required by infants, brewers' yeast was used instead of a mixture of the synthetically available components of this vitamin complex. The amino acids derived from this source can be approximated from the literature (Carter and Phillips, '44). The nitrogen content of each diet preparation was determined by the micro-Kjeldahl method and the daily N intake of the infants was calculated from the consumption records. The diets were tested in 8 normal male infants (1.5 to 9.0 months of age) for three or 4 consecutive 7-day periods. The infants were weighed daily during the course of the study.

Collection of the excreta was omitted on week ends. Nitrogen retention data were obtained by subtracting from the daily N intake the total N output as found by micro-Kjeldahl analyses of the daily 24-hour urine collections and pooled feces for the period. The urinary amino acid output was measured by chemical, microbiological and chromatographic methods; namely, methionine (Albanese, Frankston and Irby, '44); cystine (Sullivan and Hess, '36), tryptophan (Greene and Black, '44), tryrosine and phenylalanine (Albanese et al., '46; Albanese and Wagner, '44); and these and other amino acids by paper photochromatography (Albanese and associates, '51).

The effect of the diets on the blood protein was ascertained by analysis of blood samples collected on the last day of each diet period. The hemoglobin content of these specimens was measured photocolorimetrically by the acid hematin method. The total plasma proteins, albumin/globulin ratios and non-protein N were determined by techniques previously described by some of the present authors (Albanese et al., '43).

Results. The nitrogen retention and body weight changes induced by the evaporated milk (EM) and the bovine plasma digest (5 BP and 15 BP) diets indicate that by these criteria the three diets are of equal nutritional quality (table 2).

TABLE 2

Biological value of evaporated milk (EM) and bovine plasma hydrolysate formulae (BP) for the normal male infant

SUBJECT	DIET	TOTAL N INTAKE	DAILY BODY WEIGHT CHANGE	NITROGEN RETENTION	FREE URINARY AMINO N	TOTAL PLASMA PROTEINS
		gm	*gm*	*mg/kg*	*mg/day*	*gm %*
J. M.	EM	2.92	+ 12	88	56	6.25
6.0 months	15 BP	3.29	+ 28	116	202	6.34
3,908 gm	15 BP	3.50	+ 28	93	223	6.41
W. M.	EM	3.11	+ 23	138	68	5.92
1.5 months	15 BP	3.12	+ 40	180	217	5.95
3,327 gm	15 BP	3.39	+ 40	135	232	6.22
W. C.	EM	3.22	+ 31	266	76	5.81
3.0 months	15 BP	3.17	+ 40	264	195	6.10
3,879 gm	15 BP	3.40	+ 17	205	252	6.25
A. B.	EM	3.92	+ 28	198	70	6.14
6.5 months	15 BP	4.10	+ 34	167	180	6.44
6,740 gm	5 BP	4.87	+ 32	295	142	...
W. C. W.	15 BP	4.23	+ 28	158	258	5.44
6.0 months	5 BP	4.70	+ 30	259	202	5.68
6,045 gm	5 BP	4.81	+ 34	340	178	...
J. C.	15 BP	3.76	+ 57	164	135	6.70
9.0 months	5 BP	3.88	+ 21	155	82	...
5,663 gm	5 BP	4.12	+ 37	177	75	6.78
M. W.	15 BP	4.53	+ 37	125	248	5.92
4.5 months	5 BP	4.76	+ 37	200	136	5.51
8,250 gm	5 BP	4.81	+ 31	150	175	6.07

Most of the observed nitrogen retention values fell well within the range of those found in the literature (Albanese, '47a). In some instances the N retention induced by the 15 BP diets was above the norm, and almost twice the norm with the 5 BP diets.

It is important to point out that the amino N output on the "synthetic" diets was greater than that found on the evaporated milk diet. Analytical fractionation of this urinary component demonstrated that the excess was composed primarily of methionine, phenylalanine, isoleucine, tyrosine, cystine and threonine. The output of methionine and isoleucine was greatest on the 15 BP diet. This finding correlates with the fact that the bovine plasma digest employed in this diet was reinforced with these two amino acids. It is significant that normal and better than normal N retention and body weight gains were supported by these protein preparations, in the face of the urinary amino acid losses shown in table 2.

A comparison of the amounts of the various amino acids provided per kilogram of body weight by the evaporated milk formula and diets 5 BP and 15 BP is made in table 3. It can be seen that the 5 BP and 15 BP diets yielded a lower intake of certain amino acids than the amounts supplied by the evaporated milk formula. In most instances, however, these values were well above the estimated minimum needs for infants (Albanese, '50). The low methionine content of diet 5 BP is notable (Albanese et al., '49) but apparently not critical for the short period of the study, since the diet supported excellent nitrogen retention levels and body weight gains (table 2).

II. Parenteral assay in adults

It has long been recognized that shock resulting from acute infection, surgical or accidental trauma or other insult is followed inevitably by a significant loss of tissue protein which shortly places the patient in a marked negative N-balance. The causes for this reaction are not completely understood. Efforts to compensate for this loss by the administration orally or parenterally of protein preparations have been on the whole rather successful (Peters, '44; Elman, '47). The relative efficiency of commercially available protein prepara-

TABLE 3

Amino acid intake of infants on evaporated milk and bovine plasma hydrolysate diets and estimated minimum needs (All values are given in milligrams per kilogram of body weight)

AMINO ACIDS	EVAPORATED MILK	BREWERS' YEAST	5% BOVINE PLASMA DIGEST	15% [1] BOVINE PLASMA DIGEST	DIET 5 BP		DIET 15 BP		ESTIMATED MINIMUM NEEDS
					Total		Total		
	(a)	(b)	(c)	(d)	(c+b)	(c+b)−a	(d+b)	(d+b)−a	
Arginine	127	21	245	226	266	+ 139	247	+ 120	126
Aspartic acid	166	27	370	361	297	+ 231	388	222	166
Cystine	27	7	14	16	21	— 6	23	— 4	18
Glutamic acid	680	50	588	615	638	— 42	665	— 15	200
Histidine	63	19	111	102	130	+ 67	121	+ 58	63
Isoleucine	167	16	126	266	142	— 25	282	+ 115	90
Leucine	490	65	357	320	422	— 68	385	— 5	425
Lysine	200	32	343	310	375	+ 175	342	+ 142	170
Methionine	99	13	56	140	69	— 30	153	+ 54	86
Phenylalanine	177	20	182	140	202	+ 25	160	+ 17	169
Threonine	151	25	245	245	270	+ 119	270	+ 119	87
Tryptophan	47	6	49	47	55	+ 8	61	+ 14	30
Tyrosine	172	21	49	63	70	— 102	84	— 88	126
Valine	171	22	266	245	288	+ 117	267	+ 96	161

[1] This digest was supplemented with 12 gm of DL-isoleucine and 9 gm of DL-methionine per liter of solution.

tions in correcting the "N-diabetes" of physiological insult is the subject of much investigation and discussion.

In the present evaluation, having been satisfied by the foregoing studies that bovine plasma digests can sustain normal or better than normal N retention and body weight in infants by the oral route, we set out to determine their nutritive availability by the intravenous route in the convalescent adult. This availability was tested in terms of excess total, bound and free amino N and specific amino acids excreted in the urine. The rate of N catabolism was measured in terms of urea N output. The effect of dextrose infusions alone or in mixture with the protein digest on the nitrogen metabolism was also determined by these criteria.

Procedures. In order to make the necessary measurements, 4 successive 24-hour urine collections were obtained from convalescent adult males who were maintained on uniform diets and metabolically controlled. The first, or control, collection was made prior to intravenous alimentation. The second was made on the day of infusion of 1 l of 5% dextrose; the third on the day of infusion of 1 l of 5% protein hydrolysate; and the 4th on the day of infusion of 1 l of a solution containing 5% dextrose and 5% protein hydrolysate. The bovine plasma hydrolysate used in these prepartions was the same 5% digest employed in the preparation of diet 5 BP (tables 1 and 2). All infusions were given after the evening meal and at the rate of 1 l per 1.5 hours.

The urine samples were analyzed for various N and S constituents by the procedures previously mentioned.

Results. The data obtained on the 6 subjects of this study are shown in table 4.

Examination of the total N output of these subjects discloses that infusion of 1 l of 5% dextrose solution did not cause an increased loss of nitrogen but in 4 instances reduced the nitrogen output by 1 to 2 gm. This reduced N excretion may be attributed in part to the protein-sparing effect of the infused carbohydrate. The free and bound amino N output followed the trend set by total N levels. One subject (H.M.),

who failed to show any change in total N output as a result of the dextrose infusion, exhibited a drop in free and bound amino N excretion. These observations are in accord with

TABLE 4

Effect of dextrose and bovine plasma hydrolysate (Travamin) infusions on the urinary output of various metabolites by adult males

(All subjects received 1 l of each preparation per 1.5 hours)

SUBJECT	INFUSIONS	TOTAL N	UREA N	FREE AMINO N	BOUND AMINO N	TOTAL ORGANIC ACIDS
		gm	*gm*	*mg*	*mg*	*ml 0.1 N HCl*
A. P., 65 yrs.	None	12.7	9.0	370	869	616
	5% dextrose	12.9	10.7	340	835	746
65.3 kg	5% protein hyd.	14.8	12.1	700	1290	1169
	5% protein hyd. + 5% dextrose	12.9	11.0	988	1117	1200
J. G., 66 yrs.	None	10.0	8.8	253		553
	5% dextrose	7.8	7.1	151		442
51.6 kg	5% protein hyd.	10.6	8.4	436	1454	1075
	5% protein hyd. + 5% dextrose	7.8	7.1	600	875	869
H. M., 53 yrs.	None	5.5	5.1	336	329	590
	5% dextrose	5.5	4.4	218	296	474
49.3 kg	5% protein hyd.	9.2	6.7	668	1101	906
	5% protein hyd. + 5% dextrose	10.7	8.7	975	1085	1260
M. F., 50 yrs.	None	14.1	12.8	265	861	555
	5% dextrose	11.5	10.3	258	840	755
76.2 kg	5% protein hyd.	15.6	12.6	625	1980	1108
	5% protein hyd. + 5% dextrose	18.9	16.3	1479	2111	2110
J. S., 59 yrs.	None	10.3	9.6	248	527	643
	5% dextrose	9.6	9.0	200	481	680
67.0 kg	5% protein hyd.	11.0	9.4	850	990	1228
	5% protein hyd. + 5% dextrose	12.0	11.1	438	1092	907
J. H., 33 yrs.	None	11.5	10.2	285		859
	5% dextrose	10.2	9.5	269		956
70.2 kg	5% protein hyd.	17.3	14.2	722	1707	1505
	5% protein hyd. + 5% dextrose	16.3	14.4	764	1446	1560

the results obtained with test oral doses of carbohydrates by Albanese and associates ('47b).

In general the availability of intravenously administered nitrogen can be deduced from differences in metabolites found in the control urines and those collected on days when the various infusions were given. The output of nitrogenous metabolites following the infusion of 5% protein hydrolysate and 5% protein hydrolysate with 5% dextrose discloses that the infused amino acids were well utilized and that in some instances utilization was improved by the presence of 5% dextrose.

Averages show that 2.6 gm of nitrogen were excreted in excess of the control figure. Of this 18% appeared in the urine as free amino N and 27% as bound amino N. The remainder (1.5 gm) was present as urea, indicating actual metabolism of this moiety. Analytical partition disclosed the excess amino N to arise from increases in the output of cystine, methionine, histidine, phenylalanine, threonine and tyrosine. The excretion of tryptophan and inorganic S was not increased as a result of the infusions.

It is significant that the output of total organic acids followed the trends set by the amino N excretions. The ratios of total organic acids to free amino N, and total organic acids to total amino N, were not increased by the infusions. This suggests that the extra amino N did not include abnormal amounts of glutamic or aspartic acid. This is of interest since nausea and vomiting, which sometimes accompany infusion of protein hydrolysates containing large amounts of glutamic acid, were not experienced by any of our subjects.

DISCUSSION

It appears from the results obtained in this study that of the 8.0 gm of bovine plasma digest N infused in the adult males about 1.1 gm or 13.7% escaped utilization in the form of free and bound amino N. The losses ranged from 9.4 to 18.4% of the amino acid infusion. This variation may be attributable to differences in rate of infusion (Cox and Muel-

TABLE 5

Human utilization of intravenously administered protein hydrolysate

INVESTIGATORS	PROTEIN PREPARATION	NITROGEN INFUSED	VOLUME OF INFUSION	TOTAL AMINO N WASTAGE	URINE COLLECTION PERIOD
		gm	*ml*	%	*hours*
Present	5% Bovine plasma digest (Travamin)	8	1,000	9.4–18.4	24
	5% Bovine plasma digest + 5% glucose	8	1,000	9.4–18.3	24
Eckhardt et al. ('48)	Panc. digest of casein (5% Amigen)	9	1,000	17.7	24
	10% Mixture of amino acids (Merck)	7.5	500	7.8–9.5	24
Smyth and associates ('48)	Panc. digest of casein (10% Amigen)	5.4	500	1.6–19.7	4
	Acid hydrolysate of casein + tryptophan (15% Parenamine)	5.4	500	1.9–13.7	4
	10% Mixture of amino acids (Merck)	5.4	500	0.9–7.5	4
Christensen and co-workers ('47)	Limited acid hydrolysate of fibrin + 5% glucose	7.2	485–1,000	17.3–21.0	6
	Partial acid hydrolysate of fibrin (Aminosal)	7.2	1,000	5.5–11.0	6
Christensen ('50)	Complete acid hydrolysate of fibrin	5.4	1,000	5.2–7.1	4
	Enzymatic digest of bovine plasma proteins	6.1	1,000	17.9	4
	Enzymatic digest of bovine plasma proteins	3.1	500	15.5	4

ler, '46) or the nutritional states of the subjects (Allison, '48). Since in this study the rate of infusion was constant, the variations must be attributed to differences in the tissue needs of the subjects. This inference is supported by a retrospective consideration of the subjects.

A comparison of available data on total amino N losses incurred by intravenous alimentation with various protein hydrolysate and amino acid preparations discloses that the losses found with the bovine plasma digest were not excessive. In general the total amino N wastage is higher with enzymatic digest of proteins. The wastage of available peptides is about 30% of those infused, regardless of their concentration in the preparation. However, the nutritional value of these products cannot be justly appraised by this criterion. It can be argued with some validity that the retained fraction of these peptides (70%) may prove nutritionally more valuable than an equal amount of the free amino acids, since the body is spared a synthetic effort at a time of metabolic duress. Evidence for this opinion can be adduced from reports (Woolley, '45, '46; Womack and Rose, '46; Albanese and Irby, '43; Albanese, '47) which indicate that diets made with intact proteins or peptide containing protein digests have a higher nutritional value than those formulated with mixtures of amino acids or acid hydrolysates of proteins.

SUMMARY

It has been found that the nitrogen retention and weight gains of male infants maintained on a "synthetic" milk diet in which an enzymatic digest of bovine plasma [3] constituted the principal source of protein were equal, if not superior, to those obtained on an evaporated milk formula fed at the same fluid, caloric distribution and nitrogen levels. The nutritional value of the hydrolysate was not improved by reinforcement with isoleucine and methionine. Tests of the digest given parenterally to adult males showed an average utilization of 86.3% of the infused nitrogen. The possibility that

[3] See footnote 2, page 281.

the nutritional value of N derived from sources which include peptides may be greater than that of an equivalent amount of N in the form of a mixture of free amino acids is discussed.

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THE ENERGY EXPENDITURE OF BOYS AND GIRLS 9 TO 11 YEARS OF AGE (1) WASHING AND WIPING DISHES, (2) BOYS ENGAGED IN CARPENTRY, AND (3) GIRLS SEWING

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This paper is a report of further studies of the energy expenditure of children. In an extensive survey of the activities of boys and girls 9 to 11 years of age, it was found that they commonly assisted at home with the washing and wiping of dishes. Carpentry was also found to be a popular activity, particularly with the boys, while sewing was more frequently engaged in by the girls. Many children carry on these activities at school as well as at home. Since the energy expenditure of boys and girls performing these activities in a respiration chamber has not previously been determined, this investigation was undertaken.

EXPERIMENTAL

Subjects

Eighteen children (10 boys and 8 girls) served as subjects for washing and wiping dishes, while 10 boys served as sub-

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jects for the study of the energy expenditure for carpentry and 9 girls for sewing. Some of the children in the study came from a home for children and attended a public school, but the majority of the children were living at home with their parents and attended either a private or parochial school.

All of the subjects were healthy, normal boys and girls as far as could be judged from their personal appearance, growth records, and health examinations as reported by physicians and on file in their respective homes and schools. The deviations from predicted weight for height and age were within -9.7 to $+14.5\%$, using the Baldwin-Wood standards (see table 1).

Basal metabolism determinations were made on each subject as described by Taylor et al. ('48a, b, '49). The average results are presented in table 1 for each group of subjects. In all cases the deviations of the basal metabolisms from two or more of the prediction standards currently used for normal children were within the generally accepted range of $\pm 15\%$.

PROCEDURE

The open-circuit respiration chamber described by Taylor, Lamb, Robertson and MacLeod ('48a) was used for these studies. As in previous investigations, the children were studied in pairs, two boys or two girls serving as subjects at a time. The carbon dioxide produced was determined by means of the Carpenter modification of the Haldane gas analysis apparatus. The total amount of carbon dioxide was divided by two to obtain the average carbon dioxide production per child and the corresponding heat production calculated, assuming a respiratory quotient of 0.82. Determinations on children in this laboratory in the morning under standard basal metabolism conditions and in the afternoon after school, when no food had been eaten since a light noon meal, have repeatedly shown agreement well within the limits of experimental error, thus justifying the use of 0.82 as the average

TABLE 1

Average weight, height, deviation from predicted weight and basal metabolism of subjects

TABLE 1

Average weight, height, deviation from predicted weight, and basal metabolism of subjects (Figures in parentheses show range)

	WEIGHT	HEIGHT	DEVIATION FROM PREDICTED WEIGHT [1]	BASAL METABOLISM WITH STANDARD ERROR OF THE MEAN CALORIES PER			
				24 hr.	kg/hr.	m^2/hr.[3]	cm of ht./ 24 hr.
	kg	*cm*	%				
Boys, [2] **Ages 8 yr. 8 mo. to 11 yr. 0 mo.**							
Group I							
10 boys serving as subjects for washing and wiping dishes	36.6 (28.7–46.2)	143.2 (136.0–153.3)	+ 3.0 (— 7.9–+ 14.5)	1289 ± 38	1.49 ± 0.06	44.5 ± 1.0	9.0 ± 0.2
Group II							
10 boys serving as subjects for carpentry	33.5 (29.5–44.1)	141.1 (129.7–150.6)	— 2.3 (— 9.7–+ 12.0)	1275 ± 29	1.61 ± 0.06	46.4 ± 1.0	9.0 ± 0.1
Girls, [2] **Ages 8 yr. 9 mo. to 10 yr. 11 mo.**							
Group I							
8 girls serving as subjects for washing and wiping dishes	33.6 (29.3–41.1)	138.7 (126.8–149.6)	+ 1.1 (— 8.3–+ 11.7)	1190 ± 29	1.48 ± 0.04	43.5 ± 0.8	8.6 ± 0.1
Group II							
9 girls serving as subjects for sewing	35.5 (29.3–50.0)	140.6 (126.8–156.0)	+ 2.4 (— 8.3–+ 11.7)	1228 ± 46	1.46 ± 0.04	43.5 ± 0.7	8.7 ± 0.2

[1] Deviations from Baldwin-Wood standards.

[2] Many of the same boys — or girls — were subjects for each activity. In cases where they outgrew the age range or were unavailable before sufficient tests on a given activity were obtained, additional subjects were included.

[3] Surface areas were obtained from the DuBois surface area chart.

respiratory quotient and 2.996 as the Calorie equivalent per gram for the carbon dioxide produced.

Each of these activity studies was preceded by a 10-minute rest period in the chamber, during which the children sat quietly playing Chinese checkers or drawing. The two-way speaking system described by Taylor, Pye, Caldwell and Sostman ('49) was used to give the children special instructions during the test as needed. All activity studies were made in the afternoon after school, the children not having had food since a light noon meal.

Washing and wiping dishes. In the case of washing and wiping dishes, the experimental period lasted 10 minutes. This was long enough to obtain a satisfactory sample of carbon dioxide for analysis but not long enough for the children to become bored with the activity.

The customary dishpans were replaced in this study by wire mesh baskets. They were placed on wooden towel bars which were supported on legs at a convenient height so that they could serve as tables. The baskets were wired to the towel bars to prevent tipping or other accidents. This arrangement permitted a good circulation of the air within the chamber at all times, and avoided the possibility of the formation of pockets of carbon dioxide. Each child had his or her own equipment. Dishes, glasses, and silver typical of those used by a small family were placed in one of the two baskets for each child. Each article was first "washed" with a dish mop and then placed in the second basket and "wiped" with a towel. When all articles were "washed" and "dried," they were returned to the first basket and the procedure was repeated. Children of this age like to "make believe" and the fact that they actually had no water did not bother them. Before entering the chamber the boys or girls were given a demonstration and instructions.

The validity of this procedure of washing and wiping dishes without soap and water was checked by making a few determinations with the Benedict Knapsack apparatus, the children actually working at a sink using soap and water. In these

comparative tests, the energy expenditure was determined by measuring the oxygen consumption as described by Benedict and Parmenter ('28). The results of these tests fell well within the range of the results obtained in the respiration chamber. The details of the use of the Knapsack apparatus for studies with children will be discussed in a forthcoming report from this laboratory.

Carpentry. The experimental period for carpentry lasted 20 minutes. The longer period enabled the boys to construct a simple wooden toy which they were interested in making and at the same time provided ample opportunity to obtain a good sample of carbon dioxide. Before entering the chamber the boys were shown toy models, given a short demonstration, instructions, and the necessary tools and materials.

In the chamber the boys stood and worked at stools of convenient height instead of a carpenter bench. Their equipment was kept in small utility pans. They made simple wooden trains, boats, cars, guns, airplanes, bridges, and so forth, which they were permitted to take home.

Sewing. The experimental period for sewing also lasted 20 minutes, enabling the girls to make sufficient progress on their bean bags or stuffed animals to satisfy them. Before entering the chamber they were given a demonstration and the younger girls were given colorful gingham for bean bags, while the older girls were given gingham stamped with a pattern to be embroidered for stuffed animals. They were also given embroidery cotton, needles, thread, scissors, and thimble which they kept in a small tray. The bean bags were sewed with a running stitch and the animals were embroidered according to the pattern which had been stamped on the gingham. The girls thoroughly enjoyed this activity.

RESULTS AND DISCUSSION

The results of these studies, expressed as the average energy expenditure for each activity in terms of Calories per kilogram per hour and per centimeter of height per hour, are given in table 2.

Washing and wiping dishes

The average energy expenditure for the boys washing and wiping dishes was 2.92 ± 0.10 Cal. per kilogram per hour or 0.75 ± 0.03 Cal. per centimeter of height per hour, and of the girls 2.61 ± 0.10 Cal. per kilogram per hour or 0.67 ± 0.03 Cal. per centimeter of height per hour. Compared with the average basal energy expenditure in terms of Calories per kilogram per hour, the percentage increase for the boys was 96.0%,

TABLE 2

Average energy expenditure of children 9 to 11 years of age (1) washing and wiping dishes, (2) boys engaged in carpentry, and (3) girls sewing (including the basal metabolism)

	WASHING AND WIPING DISHES		CARPENTRY	SEWING
	Boys (12 cases)	Girls (12 cases)	Boys (14 cases)	Girls (13 cases)
CO_2 produced/child/hr. (gm)				
Mean ± S.E.[1]	35.41 ± 1.20	30.36 ± 1.11	40.70 ± 1.41	21.83 ± 0.70
C.V.[2] (%)	11.8	12.7	13.0	11.5
Cal./kg/hr.				
Mean ± S.E.	2.92 ± 0.10	2.61 ± 0.10	3.58 ± 0.12	1.85 ± 0.06
C.V. (%)	12.7	13.1	12.3	11.3
Cal./cm ht./hr.				
Mean ± S.E.	0.75 ± 0.03	0.67 ± 0.03	0.87 ± 0.03	0.47 ± 0.01
C.V. (%)	11.0	14.0	10.8	12.0

[1] Standard error.

[2] Coefficient of variation.

for the girls, 76.4%. The boys showed a slightly higher energy expenditure than the girls for this activity, which is in line with the findings of previous reports from this laboratory. The coefficients of variation were, respectively, 12.7% and 11.0% for the boys and 13.1% and 14.0% for the girls. The small differences between the coefficients of variation and standard errors of the mean for the boys and the girls are insignificant.

In general both the boys and girls were quite cooperative and carried out the activities in much the same way as they would have had they actually been helping at home with the washing and wiping of dishes.

It is interesting to note that the boys and girls in this investigation had an average energy expenditure per kilogram of body weight for dishwashing approximately 39% above the average generally accepted for adults as given by Rose (Taylor and MacLeod, '49).

Carpentry

The average energy expenditure of the boys doing light carpentry work was 3.58 ± 0.12 Cal. per kilogram or 0.87 ± 0.03 Cal. per centimeter of height per hour. The percentage increase above the basal energy expenditure in terms of Calories per kilogram per hour was 122.4%. This activity shows an energy expenditure for the boys somewhat higher than that for the washing and wiping of dishes. The coefficients of variation, 12.3% and 10.8%, are in line with the variations found for other activities. The boys were interested in this activity and worked continuously throughout the period, using the various tools provided in a consistent fashion in the construction of the wooden toys.

Although the carpentry in which the boys in this investigation engaged should be considered light carpentry, the energy expenditure per kilogram of body weight was slightly higher than that generally accepted for men doing heavy carpentry. See the table in Rose's Laboratory Handbook for Dietetics cited above. Bedale ('23) in England, using a Douglas bag, found that boys 11 years of age doing shopwork expended 5.09 Cal. per kilogram per hour,[3] while 9-year-old boys expended 3.75 Cal. per kilogram per hour.[4] These energy expenditures are somewhat higher than those for the boys in this study, which may be accounted for by heavier work,

[3] Calculated from data given.

[4] See footnote 3.

a cooler climate in England, and the fact that the measurements were made at varying intervals of time after the last intake of food.

Sewing

The average energy expenditure for the girls sewing was 1.85 ± 0.06 Cal. per kilogram per hour or 0.47 ± 0.01 Cal. per centimeter of height per hour. In terms of Calories per kilogram per hour the percentage increase above the basal energy expenditure was 26.7%. The energy expenditure for this activity was considerably lower than that for the girls washing and wiping dishes and very close to the energy requirements for girls sitting singing as reported by Taylor, Pye, Caldwell and Sostman ('49). The coefficients of variation are, respectively, 11.3% and 12.0%. The girls in this investigation had a higher energy requirement for hand sewing than that found by Benedict and Johnson ('19) for women, the difference amounting to about 23%. This is in line with other studies demonstrating the higher energy requirement of children per kilogram of body weight. Bedale ('23), using a Douglas bag, studied girls weaving and found that the energy expenditure of 11-year-old girls was 3.84 Cal. per kilogram per hour and of 10-year-old girls 2.54 Cal. per kilogram per hour.[5] These higher energy expenditures of Bedale's girls may be accounted for by the fact that the activity of weaving is more strenuous than the sewing in which the girls in this laboratory engaged. Also, as stated above, the cooler climate and the time of the determination with respect to the last intake of food may have affected the energy expenditure.

SUMMARY

Twenty-four determinations of the energy expenditure for washing and wiping dishes were carried out in a respiration chamber. The subjects were 10 boys and 8 girls, 9 to 11 years of age, 12 determinations being made on the boys and 12 on the girls. The results are reported as the total expenditure,

[5] See footnote 3, page 301.

i.e., inclusive of the basal metabolism. The average expenditure by the boys was 2.92 Cal. per kilogram of body weight per hour or 0.75 Cal. per centimeter of height per hour, and by the girls 2.61 Cal. per kilogram per hour or 0.67 Cal. per centimeter per hour. These results per kilogram of body weight are, respectively, 96.0% and 76.4% above the basal energy expenditure.

Fourteen determinations were made of the energy expenditure of 10 boys, 9 to 11 years of age, doing carpentry work in the chamber. The average expenditure was 3.58 Cal. per kilogram per hour or 0.87 Cal. per centimeter per hour. In terms of body weight the increase was 122.4% above the basal energy expenditure.

Thirteen determinations of the energy expenditure of 9 girls, 9 to 11 years of age, while sitting sewing in the respiration chamber were carried out. The average expenditure was 1.85 Cal. per kilogram per hour or 0.47 Cal. per centimeter per hour. In terms of body weight the increase was 26.7% above the basal energy expenditure.

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THE INFLUENCE OF VITAMIN B_{12} ON THE UTILIZATION OF CHOLINE PRECURSORS BY THE CHICK

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It was previously reported that the choline requirement of the chick is significantly decreased by supplementation of the diet with vitamin B_{12} (Schaefer, Salmon and Strength, '49), and that the nutritional requirements for choline, folacin and vitamin B_{12} are interrelated (Schaefer, Salmon, Strength and Copeland, '50). The early studies on the nutritional requirements for choline, choline precursors, and methylating compounds that were conducted prior to the isolation of vitamin B_{12} have been reviewed by Jukes ('47). Since many of the experimental diets used in earlier work contained possible sources of vitamin B_{12}, it was desirable to recheck the role of choline percursors in the promotion of growth and the prevention of perosis in the chick by use of diets low in vitamin B_{12} in comparison to diets supplemented with crystalline vitamin B_{12}.

The purpose of the present paper is to present data indicating that vitamin B_{12} is involved in the utilization of choline precursors for choline synthesis.

EXPERIMENTAL

The experimental conditions and techniques were those used in this laboratory in previous studies (Schaefer et al., '49). Day-old Leghorn chicks of the Auburn strain were

divided into three weight classes ranging from 30 to 38 gm. The selected chicks were then distributed, 8 to 12 per group, so that the average weight in each group was about 34 gm. The basal diet was composed, in per cent, of extracted peanut meal 30,[1] sucrose 36.9, extracted casein 6,[2] L-cystine 0.1, cod liver oil 1, lard 19, and minerals 7.[3] Vitamin supplements, in milligrams per kilogram of diet, were as follows: thiamine 3, pyridoxine 4, riboflavin 6, calcium pantothenate 20, niacin 50, inositol 1,000, folacin 2, biotin 0.25, alpha-tocopherol 25, alpha-tocopherol acetate 25, and 2-methyl-1, 4-naphthoquinone 5. The basal diet furnished a maximum of 0.003% choline and 0.3% methionine. The crystalline vitamin B_{12} was in the form of a sodium chloride triturate wherein 1 mg supplied 1 μg of vitamin B_{12}. When the diet was supplemented with vitamin B_{12}, it was added at a level of 30 μg per kilogram.

RESULTS

The inability of betaine, methionine, or methylaminoethanol to replace choline for the promotion of growth and prevention of perosis

Chicks fed choline-low diets supplemented with adequate amounts of vitamin B_{12} and folacin were unable to utilize betaine HCl, DL-methionine, or methylaminoethanol, fed individually, as complete replacements for the dietary choline required for growth and prevention of perosis (table 1). The activity of dimethylaminoethanol approached that of choline.

When betaine HCl was added to the diet already supplemented with vitamin B_{12} and 0.05% choline chloride, the betaine was still unable to satisfy the choline requirement of the chick. Betaine had a beneficial effect, but only in so far as three moles replaced one mole of choline. This indicates that with the diet employed the choline requirement, per se, was greater than 0.05%.

[1] Engel, R. W., J. Nutrition, 1948, *36:* 739.

[2] Salmon, W. D., J. Nutrition, 1947, *33:* 155.

[3] Schaefer, A. E., W. D. Salmon and D. R. Strength, Proc. Soc. Exp. Biol. Med., 1949, *71:* 202.

TABLE 1

The effect of choline and choline substitute compounds upon growth, perosis and mortality in chicks fed the basal diet (4-week experimental period)

COMPOUNDS TESTED	CHOLINE Cl [1] EQUIVALENT	VITAMIN B_{12} [2]	NO. OF CHICKS	AVE. WT. GAIN	PEROSIS	MORTALITY
	%			gm	%	%
Choline Cl (0.10%)	0.10	+	27	207	0	0
Choline Cl (0.05%)	0.05	+	22	122	60	0
Betaine HCl (1.98%)	0.60	+	11	95	100	10
DL-methionine (1.92%)	0.60	+	19	70	100	11
Methylaminoethanol (0.161%)	0.10	+	11	55	..	0
Methylaminoethanol (0.966%)	0.60	+	24	0	..	100
Dimethylaminoethanol (0.096%)	0.10	+	19	166	0	0
Choline Cl (0.05%) + betaine HCl (0.055%)	0.067	+	10	127	10	0
Choline Cl (0.05%) + betaine HCl (0.165%)	0.10	+	11	166	40	9
Aminoethanol (0.044%) + betaine HCl (0.33%)	0.10	+	10	94	100	10
Aminoethanol (0.044%) + DL-methionine (0.32%)	0.10	+	11	98	100	0
Methylaminoethanol (0.054%) + betaine HCl (0.22%)	0.10	+	30	191	5	0
Methylaminoethanol (0.054%) + betaine HCl (0.33%)	0.13	+	11	206	0	0
Methylaminoethanol (0.054%) + betaine HCl (0.33%)	0.13	0	22	100	25	50
Methylaminoethanol (0.054%) + DL-methionine (0.21%)	0.10	+	22	195	5	0
Methylaminoethanol (0.054%) + DL-methionine (0.21%)	0.10	0	18	86	50	66
Dimethylaminoethanol (0.127%)	0.13	+	19	183	0	0
Dimethylaminoethanol (0.127%)	0.13	0	10	47	..	80
Dimethylaminoethanol (0.288%)	0.30	0	10	46	..	60

[1] The choline equivalent was calculated on the basis of methyl content with the exception of betaine, in which 3 moles of betaine = 1 mole of choline.

[2] Crystalline vitamin B_{12} added at a level of 30 μg per kilogram of diet.

The importance of vitamin B_{12} in the utilization of methylaminoethanol and methyl donors

Vitamin B_{12} appears to be involved in the synthesis of choline from methylaminoethanol in the presence of methyl donors such as methionine or betaine. This was indicated by the high incidence of perosis or mortality, or both, and the poor weight gain in chicks fed the basal diet supplemented with methylaminoethanol plus DL-methionine or betaine, without a dietary supplement of vitamin B_{12}, and the ability of these combinations to replace choline when vitamin B_{12} was added to the diet (table 1).

Even in the presence of an adequate dietary source of vitamin B_{12} and methyl donors, the chick was unable to utilize aminoethanol as a precursor for choline synthesis for the prevention of perosis and promotion of growth. In the absence of vitamin B_{12}, dimethylaminoethanol was not utilized for the formation of choline and appeared to be toxic (table 1).

DISCUSSION

Chicks fed a choline-deficient diet supplemented with vitamin B_{12} and folacin were unable to utilize betaine, DL-methionine or methylaminoethanol, when fed individually, as complete replacement of the dietary choline required for survival, growth, and prevention of perosis. The same was true when aminoethanol was fed with betaine or DL-methionine. This confirms the earlier observations of Jukes ('41) and emphasizes that the chick is unable to methylate aminoethanol. The inability of methylaminoethanol to replace choline in the presence of vitamin B_{12} shows definitely that if the chick is able to synthesize labile methyl groups, the rate of synthesis is insufficient to meet the choline requirement. Although Stekol and Weiss ('50) and du Vigneaud, Ressler and Rachele ('50) have concluded that the rat is able to synthesize labile methyl groups, they have not shown the magnitude or the rate of such synthesis.

The near maximum growth of chicks obtained by feeding dimethylaminoethanol in the presence of vitamin B_{12} and the high mortality in the absence of vitamin B_{12} have numerous possible explanations, such as: (1) The diet contained sufficient methyl to methylate a good portion of the dimethylaminoethanol to choline and vitamin B_{12} is involved in this transfer; (2) vitamin B_{12} functions in the utilization of dimethylaminoethanol as a replacement for choline; (3) vitamin B_{12} stimulates the synthesis of methyl goups.

Vitamin B_{12} appears to be involved in the utilization by the chick of methylaminoethanol and a source of labile methyl furnished either by methionine or betaine for the synthesis of choline. The high incidence of perosis and mortality and the poor weight gain in chicks fed the basal diet supplemented with methylaminoethanol plus DL-methionine or betaine, without a dietary supplement of vitamin B_{12}, and the ability of these combinations to replace choline when vitamin B_{12} was added to the diet, indicate that vitamin B_{12} functions in the synthesis of choline via transmethylation.

In previous studies Schaefer et al. ('50) noted that chicks suffering from a double deficiency of vitamin B_{12} and choline grew more rapidly when both choline and vitamin B_{12} were supplied than when choline alone was furnished. These results indicate that the function of vitamin B_{12} is not restricted merely to choline formation. Jukes, Stokstad and Broquist ('50) demonstrated that vitamin B_{12}-deficient chicks, in contrast to normal chicks, are unable to convert homocystine into methionine when the diet is supplemented with betaine, thus indicating that vitamin B_{12} is involved in this conversion.

SUMMARY

1. Chicks fed diets supplemented with vitamin B_{12} and folacin were unable to utilize betaine, DL-methionine or methylanimoethanol, individually, in complete replacement of the dietary choline essential for growth and prevention of perosis.

2. The chick was unable to utilize aminoethanol plus betaine HCl or DL-methionine as precursors of choline even when the diet was supplemented with vitamin B_{12}.

3. The chick was able to utilize methylaminoethanol plus betaine HCl or DL-methionine as replacements for the dietary choline essential for growth and prevention of perosis only when vitamin B_{12} was added to the diet.

4. Dimethylaminoethanol as a replacement for choline prevented perosis and produced near normal growth only when the diet contained vitamin B_{12}.

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IMPROVING THE NUTRITIVE VALUE OF FLOUR

IV. THE SIGNIFICANCE OF THE USE OF ENRICHED FLOUR IN THE DIET [1]

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TWO FIGURES

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Natural foods comparable to those consumed by human beings have been used by Westerman, Linn, Templeton and Wells ('49) to evaluate the benefits of enriched flour in the diet. The diets were similar to those consumed by certain low-income groups in South Carolina (Moser, '45). The results showed that when 50% of the total calories of the diet were obtained from cereals, the animals receiving enriched flour made better growth gains than those receiving non-enriched flour.

On the basis of these results it was decided to continue the experiments and carry the growth tests through two or more generations, and also to study the reproduction and lactation performance. During the latter periods the body is undergoing a certain amount of stress and strain, which may make some of the borderline cases of deficiency diseases more acute and more easily recognized. This approach was thought to be worthwhile since certain groups of people, living on lower incomes, may use large quantities of cereal in the diets. Such a food habit may be continued from one generation to another and have considerable effect on the general health.

[1] Contribution 161, Department of Home Economics. This research was supported by a grant from the Williams-Waterman Fund and by Purnell Funds.

Through the school lunch program data were made available regarding the amounts of food consumed by some of the school children in certain communities in Kansas. A second experiment was conducted, using as a basis the amounts of food consumed by the children whose diets were high in cereal content.

EXPERIMENTAL PROCEDURE AND RESULTS

The procedure was essentially the same as that followed in previous experiments. Albino rats weighing between 45 and 55 gm and 28 days of age were placed on a B complex-free diet for two weeks to deplete their body stores of the B vitamins. At the end of the depletion period the rats were divided into 4 groups, with 8 females and 4 males in each group and equal distribution in regard to weight and litter mates. This particular distribution was made in order to evaluate reproduction and lactation performance as well as growth.

The diets used in the first experiment were based on the South Carolina data. Diet A contained enriched flour as the cereal component, while diet B contained non-enriched flour. Forty-seven per cent of the total calories in these diets came from cereals. The diets were prepared on the basis of the amount of each type of food consumed by a human being in one day. Previous experiments had shown that the rat, being a fast growing animal, needs more minerals and protein in the diet; therefore vitamin-free casein and salt mixture were added.

The flour was a commercial grade of family type flour milled from the same lot of wheat by a commercial mill and the enriching agents were added at the mill in the amounts added to flour sold commercially. The enriched flour assayed 7.28, 3.28, 44.7 and 1.21 μg per gram of thiamine, riboflavin, nicotinic acid and pantothenic acid, respectively, while the non-enriched flour assayed 1.03, 0.86, 7.93 and 1.46 μg per gram of these same vitamins.

To facilitate the preparation of the diets it was necessary to group some of the foods together. Apples were used as the fruits in the diets, dried peas and beans as the legumes, and carrots as the yellow vegetable. Spinach, cabbage, broccoli and green beans were alternated as the green vegetables, and the figures for nutritive value given in table 1 are average figures for these vegetables. The dry ingredients were weighed and mixed together. The foods that required cooking were cooked, then the wet materials were mixed thoroughly by means of a large blender and blended with the dry ingredients in such a manner that no one food could be picked out or discarded by the animals. The food was placed in glass jars and kept frozen until used.

The composition and nutritive value of the diets are shown in table 1. Calculations of nutritive value were based upon the average figures on food composition given by Sherman ('46) and Turner ('46), except for the flour. A comparison of the total nutritive value of the two diets with the amounts of these nutrients considered necessary to meet the daily requirements for human beings showed that the total calories were about average, the amounts of fat, carbohydrate and calcium were adequate, and the amounts of protein and iron were more than adequate for both diets. The differences were found in the B vitamin values. Diet B had considerably less thiamine, riboflavin and nicotinic acid than did diet A.

The growth tests were conducted over a period of 10 weeks and the results are shown in figure 1. In the first generation the animals with enriched flour in the diet made an average gain of 15 gm more than those with non-enriched flour. Their total average gain was 187 gm, as compared to 172 gm gained during the same period by the animals receiving non-enriched flour. In the second generation the difference in growth rate was much more pronounced, amounting to a total average gain of 225 gm for those with enriched flour in the diet and 164 gm for those with non-enriched, a difference of 61 gm (fig. 1). The animals on diet A also had a healthier appearance and a better growth of fur than those on diet B. These

TABLE 1

Experiment 1. Composition and nutritive value of diets A and B

(47% of total calories from cereals)

FOOD	AMOUNT	CAL.	PROT.	FAT	CHO	Ca	Fe	VIT. A	THIAMINE	RIBO.	NIACIN	VIT. C
	gm		*gm*	*gm*	*gm*	*gm*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*
Enriched flour	316	1,122	34.1	2.8	239.8	0.05	9.2		2.31	1.04	14.2	
Non-enriched flour	316	1,122	34.1	2.8	239.8	0.05	2.2		0.32	0.28	2.5	
Sugar	33	131			32.8							
Fat	53	388		42.9				1,776				
Meat	50	142	8.4	12.0			1.3	15	0.29	0.10	2.3	
Dried milk	34	169	8.8	9.1	13.0	0.32	0.2	476	0.10	0.50	0.2	
Eggs	13	21	1.6	1.5	0.9	0.01	0.4	172	0.02	0.05		
Legumes	12	42	2.7	0.2	7.4	0.01	1.0	44	0.08	0.04	0.3	
Potatoes	154	131	3.1	0.1	29.4	0.02	1.1	31	0.17	0.06	1.8	25
Carrots	48	22	0.6	0.2	4.5	0.02	0.3	4,580	0.03	0.03	0.2	3
Green vegetables	76	25	1.8	0.2	0.4	0.06	1.1	2,922	0.07	0.11	0.5	46
Apples	96	61	0.3	0.4	14.3	0.01	0.3	77	0.04	0.02	0.2	6
Salt mixture	4					0.56	14.5					
Casein (vit.-free)	30	120	30.0									
Salt	5											
Diet A												
Enriched flour	924	2,374	91.4	69.4	343	1.06	29.4	10,093	3.11	1.95	19.7	80
Diet B												
Non-enriched flour	924	2,374	91.4	69.4	343	1.06	22.4	10,093	1.12	1.19	8.0	80

results indicate that when enriched flour provided 47% of the total calories in the diet, the B vitamins and iron had a beneficial effect upon the growth rate of the rats. Since the greatest difference was noted in the second generation, and if an analogy may be drawn to the human dietary, perhaps the benefits of enriching flour may appear to a greater extent in succeeding generations than in the present one.

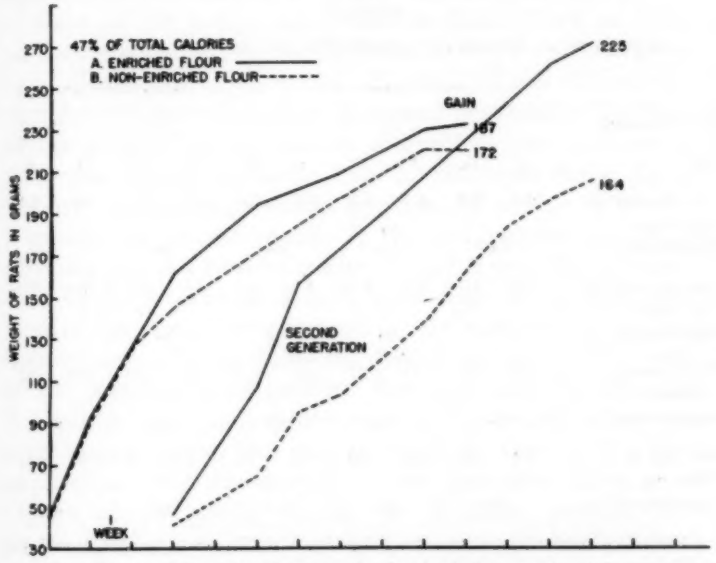

Fig. 1 Curves showing growth of animals in experiment 1.

After the growth test was completed the rats were bred. Each pregnant female was placed in an individual cage and after the young were born they were weighed and counted at one day of age and weighed weekly for 4 weeks thereafter. The young were weaned at 26 days and the females were bred again for a total of three breeding periods.

The reproduction and lactation performances are summarized in table 2 for both the first and second generation animals. All the females of the first generation on both diets

produced litters from the first mating. Those on diet A, containing enriched flour, averaged more young per litter than did the females on diet B, containing non-enriched flour. This was also true of the average number weaned per litter. The females on diet A weaned 88% of their young, while those on diet B weaned 72%. The slightly higher weights of the young on diet B at weaning may have been due to the

TABLE 2

Experiment 1. Summary of reproduction and lactation performance

	FIRST GENERATION						SECOND GENERATION			
No. of mating	1		2		3		1		2	
Diet	A	B	A	B	A	B	A	B	A	B
Ave. no. young per litter	8.6	6.5	5.5	4.3	4.0	5.6	9.0	6.0	9.0	6.0
Significance	ns'[1]		ns		ns		ns		ns	
Ave. no. weaned per litter	7.7	4.6	5.0	3.3	4.0	5.0	7.0	5.5	8.0	5.0
Significance	*[1]		ns		ns		ns		*	
% of young weaned	88	71	91	77	100	94	90	83	93	63
Significance	ns		ns		ns		ns		ns	
Ave. wt. at weaning	50	53	61	59	52	59	50	50	54	53
Significance	ns		ns		ns		ns		ns	

[1] Analysis of variance: ns' means not significant at 5% level; * means significant at 5% level.

smaller number in the litters. The females on both diets were in good condition at the end of lactation.

From the second mating (table 2) the average number of young per litter was less than from the first mating, but again the mothers on diet A produced larger litters and weaned more of their young; 91% as compared to 77% from mothers on diet B. Also the mothers receiving diet B lost considerable weight during the lactation period and the fur of the young was rough and matted.

The results of the third mating are more difficult to explain. The females on diet A had fewer young per litter than those on diet B but they weaned all the young. The fact that the females on diet A had larger litters from the two previous matings may have affected the size of their litters. In table 2 it may be noted that the females on diet B averaged considerably fewer young from the second mating period and weaned a lesser number of young than those on diet A.

Some of the young rats from the first litters of females on both diets were kept on the same diets for a second generation growth test of 10 weeks. Figure 1 shows the results of the test; the data have been discussed previously. At the end of the growth test the second generation females were bred to second generation males kept on the same diets. In both the first and second matings the females on diet A produced a greater number of young. In the first mating the per cent of young weaned by the females on diet A was 90% as against 85% by the females on diet B, while from the second mating 95% of the young were weaned by the mothers on diet A as compared to 63% by those on diet B (table 2). The results of the breeding tests indicate that enriching the flour with B vitamins and iron enabled most of the female rats to produce larger litters; this was particularly true in the second generation. The enrichment of the flour also aided in the raising of the young during the lactation periods, as is shown by the greater number of young weaned by the females on diet A and by the fact that both the adult rats and the young on this diet were in better physical condition at the end of the lactation period.

The second experiment was based upon data made available through the school lunch program. A survey had been conducted to determine the amount of food consumed by 6th grade children in two Kansas communities. About one-fourth of the children consumed diets which were high in cereal content, with 48% of the total calories from cereals. These data were used as the basis of this experiment. The composition and nutritive value of the diets are shown in table 3. The

TABLE 3

Experiment 2. Composition and nutritive value of diets D and E

(48% of total calories from cereals)

FOOD	AMOUNT	CAL.	PROT.	FAT	CHO	Ca	Fe	VIT. A	THIAMINE	RIBO.	NIACIN	VIT. C
	gm		*gm*	*gm*	*gm*	*gm*	*mg*	*I.U.*	*mg*	*mg*	*mg*	*mg*
Enriched flour	310	1,100	33.5	2.8	235.1	0.05	9.0		2.26	1.02	14.0	
Non-enriched flour	310	1,100	33.5	2.8	235.1	0.05	2.2		0.31	0.28	2.5	
Sugar	30	120			29.9							
Fat	40	293	0.3	32.4				1,340				
Meat	87	247	14.6	20.8			2.3	26	0.56	0.16	4.1	
Milk (dried)	56	278	14.4	15.0	21.3	0.53	0.4	784	0.17	0.82	0.4	
Eggs	23	36	2.9	3.5	1.6	0.02	0.7	304	0.04	0.08		
Legumes	20	70	4.5	0.3	13.0	0.02	1.6	74	0.14	0.06	0.4	
Potatoes	110	94	2.2	0.1	21.0	0.02	0.8	22	0.12	0.04	1.3	18
Carrots	54	24	0.6	0.2	5.0	0.02	0.4	5,265	0.04	0.07	0.3	3
Green vegetables	54	18	1.3	0.1	2.9	0.04	0.8	2,076	0.05	0.08	0.3	3
Apples	54	35	0.2	0.4	8.0	0.03	0.1	43	0.02	0.01	0.1	3
Salt	5											
Diet D												
Enriched flour	843	2,315	74.5	75.6	338	0.73	16.1	9,923	3.40	2.34	20.9	27
Diet E												
Non-enriched flour	843	2,315	74.5	75.6	338	0.73	9.3	9,923	1.45	1.60	9.4	27

diets were similar except that diet D contained enriched flour and diet E non-enriched flour. A summary of the nutritive value of the diets is included in table 3 and it may be noted that the diets were essentially the same in nutritive value except for the iron and B vitamin values. Diet E, which contained the non-enriched flour, was considerably lower in these essentials than was diet D, with enriched flour making up 48% of the total calories.

Twelve vitamin B-depleted rats, 8 females and 4 males, were placed upon each diet for a 10-week growth test; the

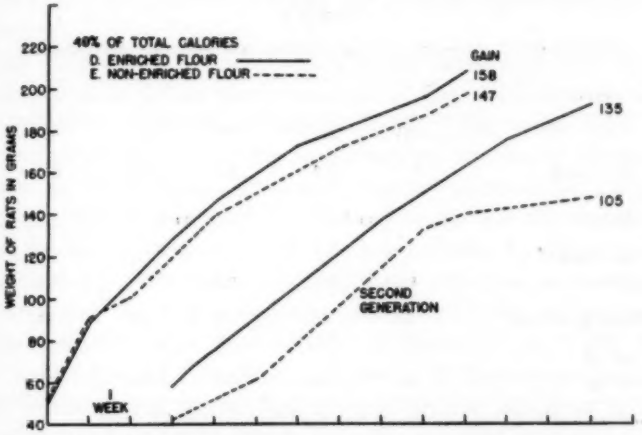

Fig. 2 Curves showing growth of animals in experiment 2.

results are shown in figure 2. The animals on diet D, containing enriched flour, showed an average total gain of 158 gm, while those on diet E, with non-enriched flour, made an average total gain of 147 gm. In the second generation growth test the differences in growth rates between the two groups were much greater (fig. 2). Those animals on diet D gained on the average 30 gm more than those on diet E during the 10-week period.

At the end of the growth test the females were bred and the test was carried through three mating periods. A sum-

mary of the reproduction and lactation performances is given in table 4. In the three reproductive periods the females on diet D, containing enriched flour, produced larger litters of young and weaned a greater number of the young than did the females on diet E, which contained non-enriched flour. The young from the mothers on diet D weighed more and had a better physical appearance than did those from the mothers on diet E. From the three mating periods the females on diet D cast 16 litters, while those on diet E cast a total of 11 litters. The total number of young born was 97 and 47 by

TABLE 4

Experiment 2. Summary of reproduction and lactation performance

NO. OF MATING	1		2		3	
Diet	D	E	D	E	D	E
Ave. no. young per litter	5	3.9	4.5	3.9	6.5	0
Significance	ns'[1]		ns			
Ave. no. weaned per litter	4.5	2	4.3	1	5.5	0
% of young weaned	72	52	72	25	84	0
Ave. wt. at weaning	42	39	51	33	45	0

[1] Analysis of variance: ns' means not significant at the 5% level.

the females on the two diets, respectively. The females on diet E took a longer time to breed than those on diet D and none of those on diet E produced litters from the third mating. Since the females consuming diet D showed better results in reproduction and lactation performance, it is to be concluded that the B vitamins and iron in the enriched flour evidently provided nutrients which were necessary during these periods of life.

In making a comparison of the data from the two experiments, it may be noted from tables 1 and 3 that the 4 diets were somewhat similar in that they contained approximately

the same amount of cereal and sugar. Diets A and B had the larger quantity of fat, vegetables and fruits, while the Kansas diets had a higher content of meat, milk, eggs and legumes, which increased the amount of the B vitamins. In the growth test (figs. 1 and 2), greater gains were made by the animals on diets A and B than those on diets D and E in both the first and second generation. This may be attributed to the additional protein and minerals in these diets, which may also account for the larger number of young weaned from the females on diets A and B. The animals needed the additional protein and minerals in the diet for growth, reproduction and lactation.

The differences between the growth rates and the reproduction and lactation performances of the animals receiving the enriched and non-enriched flour in the diets may be attributed primarily to the contribution of the B vitamins in the enriched flour. However, these results may not be be attributed to one vitamin alone, but to the interrelationships among the vitamins. It has been shown by Westerman and Bayfield ('45) that enriching flour with thiamine, niacin and iron did not improve the growth rate of rats as much as when riboflavin was also added. Williams et al. ('43) fed rats a diet low in thiamine, analagous to that consumed by some humans, and found that increasing the thiamine in the bread did not promote better growth, but that enriching the bread with both thiamine and riboflavin did induce a better growth rate. Adelson and Blake ('50) made a study of the diets of farm families in a county in Georgia and one in Ohio. Their findings showed that enrichment of flour and meal enabled nearly all the families to meet their thiamine requirements and aided considerably in meeting the requirements for riboflavin, niacin and iron.

SUMMARY

The significance of the use of enriched flour has been tested by feeding rats a combination of natural foods similar to

those consumed by human beings. Measurements of growth through two generations were made and reproduction and lactation performances were studied.

In the first experiment 47% of the total calories in the diets were obtained from enriched or non-enriched flour. These diets were also low in meat, milk, eggs and legumes. The results of the first generation growth test showed that the average total gain of the animals with enriched flour in the diet was 15 gm more than that of rats fed non-enriched flour.

In the second generation test the rats with enriched flour in the diet gained 61 gm more on the average than those fed non-enriched flour.

The reproduction and lactation performance was better among the rats receiving enriched flour, since a greater number of young were born and a greater number weaned by the females on the diets containing enriched flour.

In the second experiment 48% of the total calories in the diets came from enriched or non-enriched flour. These diets contained more meat, milk, eggs, and legumes than the diets used in the previous experiment. In the growth test the rats with enriched flour in the diet gained on the average 11 gm more in the first generation and 30 gm more in the second generation than those with non-enriched flour in the diet. The reproduction and lactation performance of the females receiving enriched flour in the diet was better than that of those receiving non-enriched flour, as was shown by the production and weaning of more young.

These experiments have produced further evidence of the beneficial effect of enriching flour.

ACKNOWLEDGMENTS

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STABILITY OF AQUEOUS AND OILY VITAMIN A ACETATE AND CAROTENE SOLUTIONS[1]

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TWO FIGURES

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One of the primary problems in animal experiments designed for studying the utilization of vitamin A or carotene is the preparation and storage of dilute solutions of the vitamin or the pro-vitamin. The preparation of solutions of pure β-carotene in which oxidation and isomerization are kept to a minimum is particularly difficult. Once prepared, the stability of such solutions during storage becomes of importance. The incorporation of anti-oxidants to stabilize vitamin A and carotene, as well as to facilitate their utilization (Hickman et al., '44; Harris et al., '44), is now common procedure.

The pertinent literature on the numerous studies of the stability of vitamin A, both in oily and aqueous media, has been cited in the recent papers of Kern et al. ('49) and Kern and Antoshkiw ('50). No reports are available concerning the stability of aqueous preparations of carotene (Tomarelli et al., '46). Stability studies of vitamin A or carotene which have been reported generally are not suitable guides for the preparation of solutions to be used for quantitative animal experimentation, for one or more of the following reasons: (a) the use of impure preparations of vitamin A or caro-

[1] Presented before the Biochemistry Section, 6th Southwest Regional Meeting of the American Chemical Society, San Antonio, Texas. December 8, 1950.

tene; (b) the use of solutions with very high vitamin A or carotene content; (c) the use of accelerated storage tests carried out at elevated temperatures; and (d) the use of anti-oxidant concentrations other than those known to be optimum for the utilization of vitamin A and carotene in the rat (Hickman et al., '44; Harris et al., '44).

The present study was undertaken to determine the stability of dilute aqueous and oily solutions of pure vitamin A acetate and β-carotene under optimum conditions of preparation and storage.

EXPERIMENTAL

Two separate studies were performed. In the first, the stabilities of vitamin A acetate and β-carotene in Tween 40 and cottonseed oil solutions, with anti-oxidants, were determined. In the second, the stabilities in aqueous Tween 40 and Tween 80 solutions, without anti-oxidants, were determined.

Crystalline vitamin A acetate [2] was used since this form of the vitamin in cottonseed oil is now the standard of the U. S. Pharmacopeia ('50). All *trans*-β-carotene was prepared from commercial 90% β, 10% α-carotene by chromatographing on a column of magnesium oxide [3] and Hy-flo Supercel (1:2). The β-carotene band was eluted with 10% ethanol in Skellysolve B, the solvent removed at 40° C. under vacuum, and the carotene crystallized from benzene and methanol. A typical batch of carotene prepared by this procedure had an absorbancy index in hexane at 453 mμ (slit = 0.041 mm) of 2520 (cell thickness = 1 cm, concentration = grams per 100 ml; National Bureau of Standards, '47). The melting point was 180° C. (corrected). Furthermore, the ratio of the absorbancy in hexane at 345 mμ to that at 453 mμ was of the order of 0.05 to 0.07. This ratio is a good criterion of the presence or absence of oxidized or isomerized carotene (Zechmeister, '44).

[2] Generously supplied by Hoffmann-LaRoche, Inc.

[3] Westvaco.

For the water solutions, Tweens (fatty acid esters of polyoxyethylene sorbitan) [4] were used as dispersants. Tween 40 (the palmitate ester) was used in the first study in preference to other Tweens for several reasons. Tween 20 (the laurate ester) does not disperse carotene as easily as do the higher fatty acid Tweens. Tween 60 (the stearate ester) has the disadvantage of becoming hard when stored at 7°C. and requires prolonged thawing, or heating, before use.

For the oily preparations, cottonseed oil [5] was used. Two anti-oxidants were employed for comparative purposes, DL-α-tocopherol [6] and nordihydroguaiaretic acid (NDGA).[7] These, together with synergists, were added to the Tween or cottonseed oil so that the concentrations in the final preparations were: 0.3% α-tocopherol with 0.3% ascorbic acid and 0.1% NDGA with 0.08% citric acid. No ascorbic acid was added to the cottonseed oil. The concentration of α-tocopherol was chosen so that 0.1 ml of the final preparation would supply approximately that amount of tocopherol (0.3 to 0.7 mg) shown by several workers (Hickman et al., '44; Harris et al., '44; Lemley et al., '47; Miles et al., '49) to give the optimum utilization of vitamin A and carotene in rats. The concentrations of NDGA and citric acid chosen have been shown by Dassow and Stansby ('49) to afford adequate protection of vitamin A in fish liver oils. In the second study, no anti-oxidants were used.

To prepare the aqueous solutions, 2 mg of vitamin A acetate or β-carotene were placed in a 20-ml glass vial and dissolved in 0.1 ml chloroform. Two milliliters of Tween warmed to 40° C. were then added with stirring, using a magnetic stirrer, followed by 8 ml of water at 40° C. The procedure for the oily solutions was similar; the sample was first dissolved in 0.1 ml chloroform and 10 ml of oil were added while stirring.

[4] Atlas Powder Co.

[5] Wesson oil.

[6] Merck.

[7] Kindly supplied by the Nordigard Corp., Chicago.

Such preparations of both vitamin A acetate and β-carotene are clear solutions. By keeping the temperature below 40° C., oxidation and isomerization of carotene are virtually eliminated.

In the first study, the preparations were stored under nitrogen. In the second study, half of the samples were kept under nitrogen and the other half under air. All vials were stored at 7° C. in the dark. At intervals, duplicate samples (0.1 to 0.2 gm) were weighed into glass-stoppered test tubes and saponified with 2 ml of 1N alcoholic KOH at 50° C. for 25 minutes. After cooling, 2 ml of water were added, the contents extracted by shaking for two minutes with 10 ml of Skellysolve B, and the tubes centrifuged. Aliquots of the extract were taken for carotene or vitamin A analyses.

Carotene was determined directly on the extract by its absorption at 453 mμ, using the Beckman Model DU spectrophotometer.

Due to the relatively high concentrations of anti-oxidants present, vitamin A could not be determined spectrophotometrically. Consequently, the extract was dried over sodium sulfate, aliquots evaporated to dryness *in vacuo*, the residue taken up in chloroform, and the vitamin A determined by the antimony trichloride method using the Coleman Junior spectrophotometer.

RESULTS AND DISCUSSION

The results of the first study (fig. 1) indicate that under the conditions used vitamin A acetate and β-carotene in dilute aqueous Tween 40 or oily solutions with anti-oxidants are stable for at least 50 days. Nordihydroguaiaretic acid did not protect either vitamin A acetate or β-carotene in a water-Tween solution as well as did α-tocopherol, although the differences are not appreciable (about 8% less vitamin A acetate and 5% less carotene remaining). The superiority of NDGA in cottonseed oil over the same anti-oxidant in water-Tween may be due to the small amounts of tocopherols naturally present in

the oil. The stability in cottonseed oil substantiates the findings of Chilcote et al. ('49) with more concentrated solutions. They found vitamin A acetate in oil to be stable for almost a year when stored under nitrogen at 5°C.

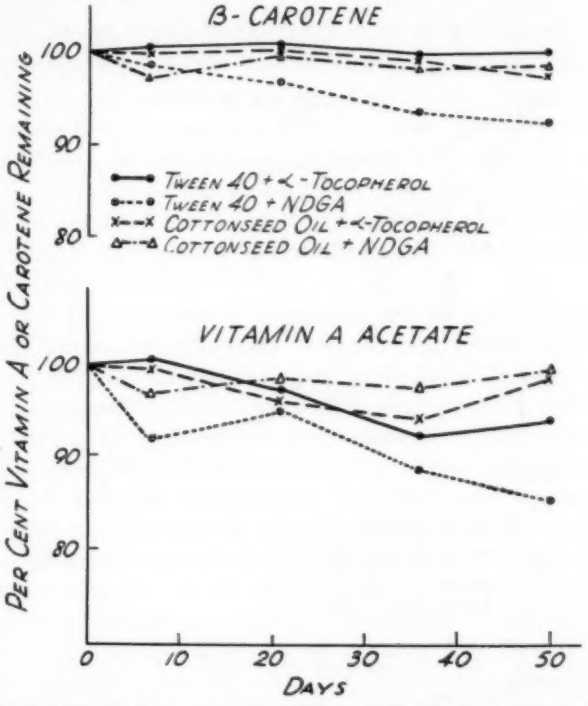

Fig. 1 Stability of dilute solutions (0.02%) of vitamin A acetate and β-carotene in water (with 20% Tween 40) and in cottonseed oil, when stored at 7°C., under nitrogen, in the presence of anti-oxidants.

Chromatographic analysis of the carotene samples (after saponification and extraction) on magnesium oxide after 75 days of storage showed the three more stable preparations to be chromatographically homogeneous, whereas the Tween-NDGA solution gave a small amount of oxidation products at the top of the column.

The results of the second study (fig. 2) show the effects of an inert atmosphere and of different Tweens on the stability of vitamin A acetate and β-carotene in aqueous solution for a 28-day storage period. Vitamin A acetate was about equally stable in Tween 40 or Tween 80 solutions without anti-oxidants, and in an atmosphere of either air or nitrogen. By a comparison of the results in figure 1 and figure 2 for

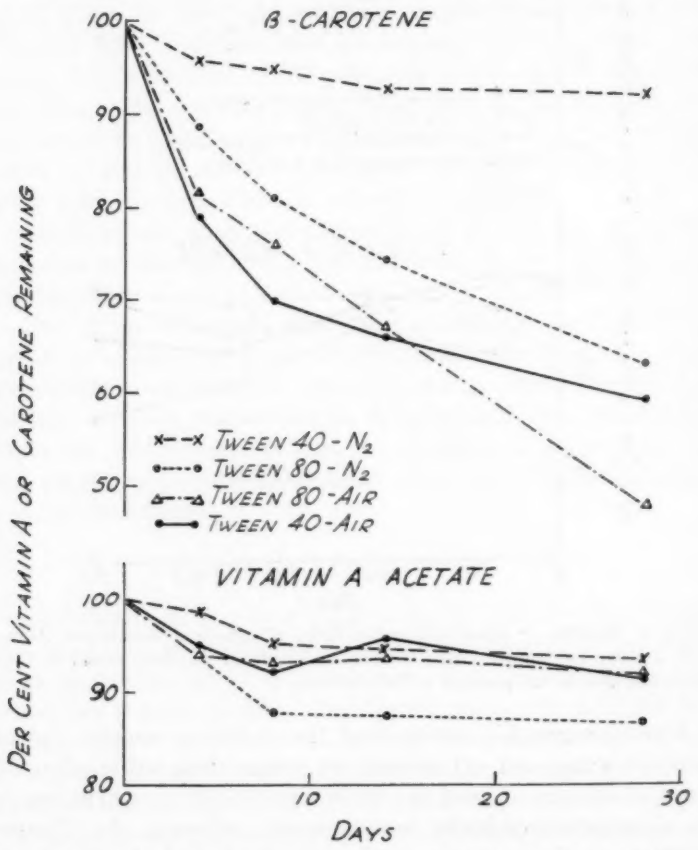

Fig. 2 Stability of dilute solutions (0.02%) of vitamin A acetate and β-carotene in water (with 20% Tween 40 or Tween 80), when stored at 7°C., under nitrogen or air, without anti-oxidants.

vitamin A acetate, it would appear that this substance is quite stable at 7°C. in aqueous solutions for moderate periods even in the absence of anti-oxidants. Kern and Antoshkiw ('50) found that the pH of aqueous preparations (using Tween 20) of vitamin A alcohol affected the stability, with an optimum pH of 5 to 9 in the absence of anti-oxidants. When tocopherols were present, however, the stability was greatest at pH 4 to 5, and considerably less at pH 7 to 9. In the present study, the pH of the aqueous solutions was similar for Tween 40 and Tween 80 without anti-oxidants (6.2 and 6.8, respectively), while the Tween 40 solutions with anti-oxidants were more acid (about 3.6). The pH effect would thus not account for the slight differences in stability in the Tween solutions noted in the first study.

The stability of aqueous β-carotene in the absence of anti-oxidants varied considerably (fig. 2). In Tween 40, under nitrogen, 92% of the carotene remained after 28 days, while in a similar preparation stored under air, only 60% remained. In Tween 80, 64% of the carotene remained under nitrogen, and only 48% after 28 days under air. These results are in agreement with the well-known fact that carotene is very susceptible to oxidation by air. A comparison of the Tween 40 and Tween 80 preparations stored under nitrogen reveals the superiority of Tween 40 for such purposes. Since the difference cannot be explained on the basis of pH, as noted above, it is probably due to the presence in Tween 80 of oxidized unsaturated fatty acids.[8] The coupled oxidation of carotene or vitamin A with fatty acids has been the subject of numerous studies (Holman, '49, '50). The results given here indicate that vitamin A acetate is much less susceptible to coupled oxidation than is carotene.

SUMMARY

Vitamin A acetate or β-carotene in dilute aqueous Tween 40 or cottonseed oil solutions with α-tocopherol or nordihydro-

[8] The Atlas Powder Co., in a private communication, states that the peroxide value of Tween 80 varies but is in the range of 6.9.

guaiaretic acid (NDGA) as anti-oxidants are essentially quantitatively retained after 50 days of storage under nitrogen at 7°C. in the dark. Tocopherol affords slightly better protection to the aqueous preparations than does NDGA. In the absence of anti-oxidants, vitamin A acetate is stable for 28 days in aqueous solutions with either Tween 40 or Tween 80, whether stored under air or nitrogen. The stability of carotene under similar conditions is high in Tween 40 stored under nitrogen, but falls rapidly in Tween 40 under air or in Tween 80 whether stored under nitrogen or air.

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